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(54) Title: ASSAYS

(57) Abstract: The invention provides a compound comprising nucleotide sequences: -5'-P-N^1-N^2-G-N^3-C-I-3' and 3'-Q-N^4-N^5-N^6-N^7-N^8-N^9-Y-J-5' wherein P and Q are any two nucleotides that can form a Watson-Crick base pair, I and J are any two nucleotides that can form a base pair, N^1 and N^4 are not both C, when N^2 is A, N^7 is not G, N^9 can only be U if N^3 is A, when N^3 is G, N^9 is A, and wherein said sequences are capable of annealing, characterised in that said compound comprises 200 or fewer nucleotides.

ASSAYS

TECHNICAL FIELD

This invention relates to assays for molecules that interact with the hepatitis C virus genome and to compounds for use in such assays.

BACKGROUND ART

Cap-independent translation of hepatitis C virus (HCV) genomic RNA is mediated by an internal ribosome entry site (IRES) within the 5'-UTR of the viral RNA (Figure 1), and inhibiting the interaction of translation initiation factors (*e.g.* Eukaryotic Initiation Factor 3 – eIF3) with the 5'UTR has been proposed as a therapeutic strategy [*e.g.* references 1, 2 and 3].

Assays for this inhibition are not, however, straightforward. Culture of HCV is very difficult and slow, so indirect assays and cell-free systems are used instead, in which translation of a reporter gene controlled by the 5'-UTR is monitored. Several reporter genes have been described. Reference 4 utilises the chloramphenicol acetyltransferase (CAT) gene fused to the HCV-1b 5'-UTR, with a subsequent assay of CAT activity indicating whether translation from the 5'-UTR was inhibited. Reference 5 describes a bicistronic construct containing two different luciferases, the first being translated in a cap-dependent manner and the second being translated, like wild-type HCV, in a cap-independent manner. The relative levels of the two luciferases gives an indication of whether the cap-independent translation was inhibited. By determining cap-independent translation in the present of test compounds, therefore, these systems offer indirect ways of following the prevention of translation initiation in the 5'-UTR.

Whilst these indirect assays are easier than direct testing of HCV in culture, they still have drawbacks. Firstly, the complete 5'-UTR is required in each case. Secondly, they rely on protein synthesis and therefore on the presence of ribosomes and free amino acids in the system, making the assays relatively cumbersome, time-consuming and expensive. Finally, indirect assays cannot, by their nature, directly monitor the eIF3/5'-UTR interaction. Positive results may, therefore, reflect an interaction of the test compound with the ribosome or with a host protein required for IRES function, rather than specific inhibition of the 5'-UTR itself.

There is thus a need for simpler, quicker and cheaper assays for monitoring 5'-UTR activity.

Specific binding of eIF3 to the HCV IRES has been reported by Sizova *et al.* [6]. Sizova *et al.* reported an enzymatic footprinting analysis of a complex formed between eIF3 and the

complete HCV IRES. In particular, eIF3 was found to protect certain domains of the HCV IRES from cleavage by RNases ONE and V_1. Such nuclease footprints may arise for a number of reasons. For example, inhibition of nuclease cleavage of the IRES may be the result of eIF3 binding at a site in the IRES which induces a conformational change in the IRES; or it may be the result of a steric shadow arising from binding of eIF3 at an adjacent or remote site in the IRES; or it may be the result of direct interaction of eIF3 with the binding site; or it may be the result of an artefact of the footprinting technique.

Kieft *et al.* [7] report the results of mutagenesis experiments in which point mutations were made at the IIIabc four-way junction and in the IIId loop. The authors report that one mutation (U228C) in the IIIabc junction results in >95% inhibition of HCV IRES activity and that the IIId loop was critical for function.

DISCLOSURE OF INVENTION

Surprisingly, it has been found that a small sub-region of the 5'-UTR, shown in generalised formula in Figure 2, is essential for the binding of eIF3 and that this sub-region can be used in an assay to assist in the identification of drugs which inhibit HCV translation initiation. Assays based on this sub-region, referred to hereafter as 'mIRES' (for 'minimal IRES') enable potential anti-virals to be screened in a cheaper and easier way. The compounds of the invention, which comprise the mIRES or mimics thereof, allow rapid assays using a small volume of material, which are also suited to parallel processing.

According to the present invention there is provided a compound comprising nucleotide sequences:-

$5'$-P-N^1-N^2-G-N^3-C-I-$3'$ and $3'$-Q-N^4-N^5-N^6-N^7-N^8-N^9-Y-J-$5'$

wherein

- P and Q are any two nucleotides that can form a Watson-Crick base pair,
- I and J are any two nucleotides that can form a base pair, preferably a Watson-Crick base pair,
- N^1 and N^4 are not both C,
- when N^2 is A, N^7 is not G,
- N^9 can only be U if N^3 is A,
- when N^3 is G, N^9 is A,

and wherein said sequences are capable of annealing, characterised in that said compound comprises 200 or fewer nucleotides.

According to a further aspect of the present invention there is provided a compound comprising nucleotide sequences:-

5'-P-N^1-N^2-G-N^3-C-I-3' and 3'-Q-N^4-N^5-N^6-N^7-N^8-N^9-Y-J-5'

wherein

- P and Q are any two nucleotides that can form a Watson-Crick base pair,
- I and J are any two nucleotides that can form a base pair, preferably a Watson-Crick base pair,
- N^1 and N^4 are not both C,
- when N^2 is A, N^7 is not G,
- N^9 can only be U if N^3 is A,
- when N^3 is G, N^9 is A,

and wherein said sequences are capable of annealing, provided that said compound is not a complete HCV genome or fragment thereof.

The compounds of the present invention comprise the mIRES of an HCV genome or comprise a structure which mimics the mIRES of an HCV genome. Preferably the compounds of the present invention comprise a structure which adopts a conformation substantially identical to a mIRES of an HCV genome and which exhibits a ligand binding pattern substantially identical to a mIRES of an HCV genome.

Standard nucleotide abbreviations are used herein: A is a nucleotide comprising an adenine base; G is a nucleotide comprising a guanine base; C is a nucleotide comprising a cytosine base; T is a nucleotide comprising a thymine base; U is a nucleotide comprising a uracil base; R is a nucleotide comprising a purine base (*i.e.* A or G); Y is a nucleotide comprising a pyrimidine base (*i.e.* C or U or T); and N is any nucleotide. Each occurrence of R, Y or N in a sequence may be the same or different. The term "W•C" refers to a Watson-Crick base paid (*i.e.* G•C, C•G, A•U, U•A, A•T, T•A).

As used herein the term nucleotide may refer to a deoxyribonucleotide or a ribonucleotide. The compounds of the invention may comprise RNA or DNA, or a mixture of both. Preferably, the compounds of the present invention are RNA.

In the compounds of the invention:

- flanking base pairs P•Q and I•J may be any Watson-Crick base pair. P•Q is preferably a R•Y base pair, particularly G•C. I•J is preferably a Y•R base pair, particularly C•G.

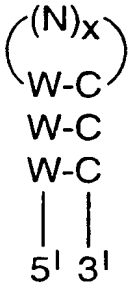
- N^1 is preferably A or G. When N^1 is A, N^4 is preferably U or, more preferably, G or A. When N^1 is G, N^4 is preferably C or, more preferably, A or U.
- N^2 is preferably A, C, G or U. When N^2 is A, N^7 is preferably U. When N^2 is C, N^7 is preferably C or U or, most preferably, A. When N^2 is G, N^7 is preferably C or U. When N^2 is U, N^7 is preferably U.
- N^3 and N^9 can preferably form an A•U base pair *i.e.* when N^3 is A, N^9 is preferably U, and when N^3 is U, N^9 is preferably A.
- N^4 is preferably A, C, G or U. When N^4 is A, N^2 is preferably A or G. When N^4 is C, N^2 is preferably G. When N^4 is G, N^2 is preferably A. When N^4 is U, N^2 is preferably A or G.
- N^5 is preferably U.
- N^6 is preferably G, U or, most preferably, A.
- N^7 is preferably A, C, G or U. When N^7 is A, N^2 is preferably C. When N^7 is C, N^2 is preferably C or G. When N^7 is G, N^2 is preferably U. When N^7 is U, N^2 is preferably C, A or G.
- N^8 is preferably C.
- Y is preferably C.

The compounds of the present invention may comprise a single molecule, for example a single stranded RNA molecule comprising both said sequences. Alternatively, the compounds of the present invention may comprise two or more, preferably two, annealed molecules, for example two single stranded RNA molecules each comprising one of said sequences and annealed to one another.

Where the compounds comprise a single molecule the 3' end of the sequence 5'-P-N^1-N^2-G-N^3-C-I-3' and the 5' end of the sequence 3'-Q-N^4-N^5-N^6-N^7-N^8-N^9-Y-J-5' may be joined by a linker which allows said sequences to anneal. Preferably, the compound comprises the sequence 5'-$(N)_a$-[P-N^1-N^2-G-N^3-C-I]-$(N)_b$-[J-Y-N^9-N^8-N^7-N^6-N^5-N^4-Q]-$(N)_c$-3' wherein N may be the same or different and are any nucleotide, a is zero or an integer from 1 to 100 (preferably $\geq$4), b is an integer from 3 to 100 (preferably $\geq$4), and c is zero or an integer from 1 to 100 (preferably $\geq$4). Preferably, a=c.

Preferably, the linker -$(N)_b$- comprises a sequence which can serve to stabilise the mIRES *i.e.* which maintains the mIRES conformation so that its structure (*e.g.* secondary and tertiary)

corresponds to that of the wild-type. The mIRES may, for example, be stabilised by a nucleotide sequence capable of forming a duplex comprising Watson-Crick base pairs, a cross-linked sequence, and/or a sequence capable of forming a secondary structure such as a loop. For example, the linker -$(N)_b$- may comprise nucleotides (RNA or DNA or both) capable of forming a duplex comprising one or more Watson-Crick base pairs adjacent to the mIRES, *i.e.* adjoining the 3'end of the sequence 5'-P-N^1-N^2-G-N^3-C-I-3' and the 5' end of the sequence 3'-Q-N^4-N^5-N^6-N^7-N^8-N^9-Y-J-5'. Preferably, the linker -$(N)_b$- comprises nucleotides capable of interacting to form from one to ten, preferably two to four, more preferably three, Watson-Crick base pairs adjacent to the mIRES. The linker -$(N)_b$- may also be capable of forming a loop structure. The linker -$(N)_b$- may, for example, comprise the structure:



wherein x is an integer from 4 to 20.

Where the compound of the present invention comprises two or more annealed sequences, the compound may comprise a first nucleic acid strand comprising the sequence 5'-P-N^1-N^2-G-N^3-C-I-3' annealed to a second nucleic acid strand comprising the sequence 3'-Q-N^4-N^5-N^6-N^7-N^8-N^9-Y-J-5'. Preferably, the compound comprises a first nucleic acid strand comprising the sequence 5'$(N)_a$-P-N^1-N^2-G-N^3-C-I-$(N)_{b1}$-3' annealed to a second nucleic acid strand comprising the sequence 3'-$(N)_c$-Q-N^4-N^5-N^6-N^7-N^8-N^9-Y-J-$(N)_{b2}$-5', wherein a is zero or an integer from 1 to 100, b^1 is zero or an integer from 1 to 100. b^2 is zero or an integer from 1 to 100. c is zero or an integer from 1 to 100. Preferably, a=c and/or $b^1=b^2$ (*e.g.* Figure 4).

Preferably, -$(N)_{b1}$- and -$(N)_{b2}$- are capable of interacting to stabilise the mIRES. The mIRES may, for example, be stabilised by nucleotides capable of forming a duplex comprising Watson-Crick base pairs, cross-linked sequences and/or a sequence capable of forming a secondary structure such as a loop. Preferably, -$(N)_{b1}$- and -$(N)_{b2}$- comprise nucleotide sequences capable of annealing to each other. For example, -$(N)_{b1}$- and -$(N)_{b2}$- may comprise nucleotides (RNA or DNA or both) capable of forming a duplex comprising one or more Watson-Crick base pairs adjacent to the mIRES. Preferably, -$(N)_{b1}$- and -$(N)_{b2}$- comprise

nucleotides capable of forming from one to ten, preferably two to four, more preferably three, Watson-Crick base pairs adjacent to the mIRES.

Preferably, $-(N)_a-$ and $-(N)_c-$ are capable of stabilising the mIRES. The mIRES may, for example, be stabilised by nucleotides capable of forming a duplex comprising Watson-Crick base pairs, cross-linked sequences and/or a sequence capable of forming a secondary structure such as a loop. Preferably, $-(N)_a-$ and $-(N)_c-$ comprise sequences capable of annealing to each other. For example, $-(N)_a-$ and $-(N)_c-$ may comprise nucleotides (RNA or DNA or both) capable of forming a duplex comprising one or more Watson-Crick base pairs adjacent to the mIRES *i.e.* adjoining the 5' end of the sequence 5'-P-N^1-N^2-G-N^3-C-I-3' and the 3' end of the sequence 3'-Q-N^4-N^5-N^6-N^7-N^8-N^9-Y-J-5'.

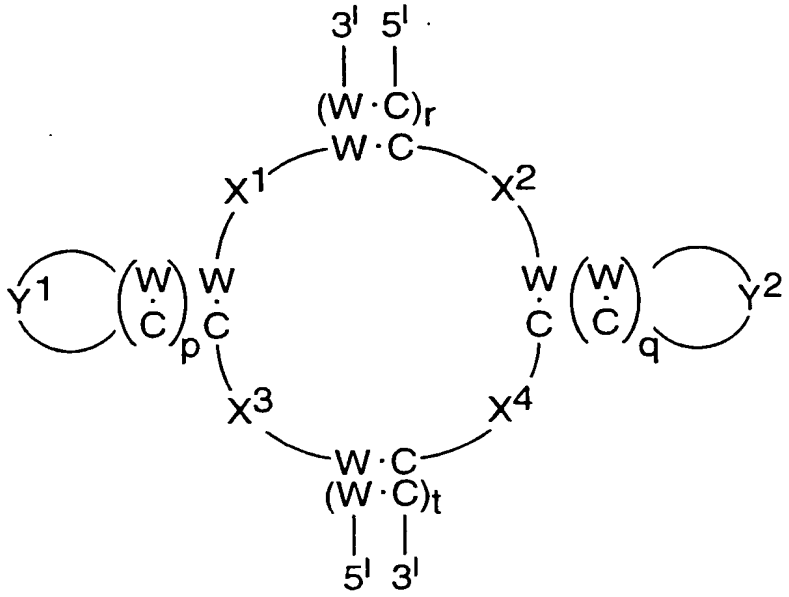
Preferably, $-(N)_a-$ and $-(N)_c-$ are capable of interacting to form from one to forty, preferably one to twenty, Watson-Crick base pairs adjacent to the mIRES. $-(N)_a-$ and/or $-(N)_c-$ may independently form a loop structure and/or may interact to form a junction structure, such as a four-way junction.

Where the compound is used in an assay based on binding to a reporter molecule, it is generally desirable to minimise the size of the compound in order to simplify preparation, purification and handling. In such circumstances the compound of the present invention preferably comprises 100 or fewer, more preferably 50 or few, more preferably 30 or fewer nucleotides. The ability to use a shortened mimic of the 5'-UTR allows simple and rapid chemical synthesis, and also aids enzymatic synthesis by, for instance, T7 polymerase. The introduction of chemical modifications is also easier.

It has been found, however, that whilst the mIRES is essential for binding of eIF3, the mIRES alone is not sufficient for binding eIF3. Accordingly, in assays based on binding of the compound to eIF3, the compound of the present invention preferably comprises 50 or more, more preferably 100 or more, more preferably 150 or more nucleotides.

The compound of the invention preferably binds eIF3 with an affinity $\geq$2% the affinity of the interaction between HCV 5'-UTR domain III (DIII) and eIF3. More preferably, the affinity is $\geq$10%, and most preferably $\geq$50%, that of the DIII/eIF3 interaction.

In a preferred embodiment particularly suitable for use in an assay based on binding of the compound to eIF3, $-(N)_a-$ and $-(N)_c-$ are capable of forming a structure comprising:



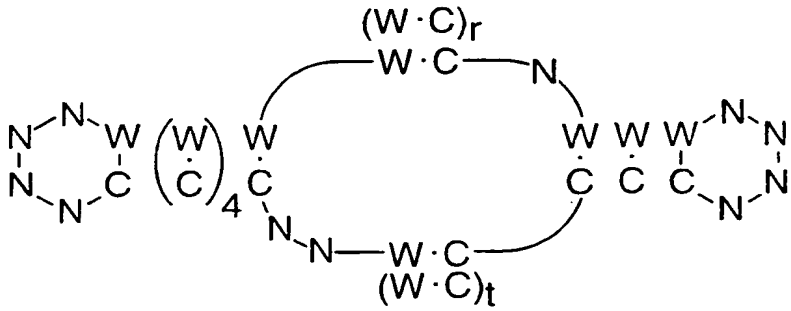
wherein each W-C may be the same or different and is a Watson-Crick base pair,

X^1, X^2, X^3 and X^4 may be the same or different and comprise from zero to four nucleotides,

Y^1 and Y^2 may be the same or different and comprise from three to ten nucleotides,

p, q, r and t may be the same or different and integers from zero to ten.

Preferably, $-(N)_a-$ and $-(N)_c-$ are capable of forming a structure comprising:



The sequences of the 5'-P-N^1-N^2-G-N^3-C-I-3' and 3'-Q-N^4-N^5-N^6-N^7-N^8-N^9-Y-J-5' regions from published HCV sequences are shown aligned in Figure 3. It will be noted, for instance, that the 5' residue of Figure 3A has been selected by nature to be G or, in a single case, A. The complementary residue is Figure 3B is similarly, therefore, either C or, in a single case, U. According to the invention, however, there is no such restriction.

Thus in preferred compounds of the invention: P is a purine; Q is a pyrimidine; I is a pyrimidine: J is a purine; N^1 is A, G or U; N^2 is A, C or U; N^3 is A; N^4 is a purine; N^5 is U; N^6

is a purine; N^7 is A, G or U; N^8 is C; N^9 is U; and Y is C. These compounds represent the sequences of Figure 3, which is an alignment of the sequences from published HCV genomes.

The sequences 5'-P-N^1-N^2-G-N^3-C-I-3' and 3'-Q-N^4-N^5-N^6-N^7-N^8-N^9-Y-J-5' employed in the present invention may comprise sequences corresponding to wild-type HCV sequences or may comprise sequences which do not consist of naturally occurring HCV sequences.

Where the sequences do consist of to wild-type HCV sequences, the sequences may be one of the 35 pairs of sequences shown in Figure 3. Preferably, the sequences are 5'-G-A-C-G-A-C-C-3' and 3'-C-G-U-A-A-C-U-C-G-5', said sequences being those from the prototype genome of strain 1a (M67463).

As defined herein the invention encompasses

1. Shortened 5'-UTR - The mIRES is much smaller than the complete 5'-UTR. Therefore in one aspect the invention provides a compound comprising sequences 5'-P-N^1-N^2-G-N^3-C-I-3' and 3'-Q-N^4-N^5-N^6-N^7-N^8-N^9-Y-J-5', wherein said sequences are capable of annealing to each other, characterised in that the molecule comprises 200 or fewer nucleotides.

2. Heterologous context - The mIRES is able to bind eIF3 and other reporter molecules in contexts other than the native 5'-UTR. The invention therefore provides the mIRES in a heterologous context, that is to say, flanked on one or both sides by sequences not found in a HCV genome. The ability to place the mIRES in a heterologous context, for instance being flanked by stem structures, allows a very stable molecule to be produced. Furthermore, only nine consecutive nucleotides from the wild-type HCV sequence are required for function.

3. Double-stranded - The wild-type HCV genome consists of a single RNA strand. Stem IIIb and the mIRES are formed from this single strand folding back on itself to form a duplex region. The identification of the mIRES allows the construction of a functional molecule from two separate nucleic acid strands. Accordingly, in this aspect, the invention provides a compound comprising a first nucleic acid strand comprising the sequence 5'-P-N^1-N^2-G-N^3-C-I-3' annealed to a second nucleic acid strand comprising the sequence and 3'-Q-N^4-N^5-N^6-N^7-N^8-N^9-Y-J-5'. The ability to form the mIRES from separate strands simplifies the chemical synthesis of the compounds of the invention. It also facilitates differential labelling of the two strands.

4. Modified mIRES - As well as identifying a minimal fragment of the 5'-UTR that is essential for binding eIF3, the mIRES has been dissected internally to determine its critical residues. The generic formula of the mIRES the subject of the present invention and is shown in Figure 2. Knowledge of this generic formula permits mutation of the mIRES whilst retaining functional binding. This allows desired sequences and/or non-wild-type sequences to be incorporated into the mIRES without losing function. Modified mIRES can be presented in the same ways as described above for the native mIRES (*i.e.* in shortened, heterologous and/or double stranded context). In this aspect of the invention, where the compound is not a complete HCV genome or fragment thereof, the sequences 5'-P-N^1-N^2-G-N^3-C-I-3' and 3'-Q-N^4-N^5-N^6-N^7-N^8-N^9-Y-J-5' should not be one of the pairs shown in Figure 3.

Production

The molecules of the invention may be produced in various ways, including transcription from DNA templates by RNA polymerase, by enzymatic replication of RNA templates, or by chemical synthesis with an automated oligonucleotide synthesiser. Suitable RNA polymerases for transcription include the bacterial polymerases T3 and T7, Sp6, and *E.coli* polymerase. Suitable RNA replicases include the replicase from Qβ RNA polymerase.

Modifications

RNA is sensitive to cleavage by cellular ribonucleases, as well as to alkaline or acid conditions. The molecules of the invention may therefore contain modifications that confer greater stability. Modifications may also be desirable to provide groups for immobilising the RNA on solid supports by covalent or non-covalent attachments. Typical modifications include modified bases and/or sugars and/or linkages. The terms "RNA molecule", "nucleotide" and "oligonucleotide" as used herein are intended to cover all such variants.

Modifications may include, but are not limited to, the following types:

a) Backbone modifications:

(i) phosphorothioates (single S substituents or any combination of two or more with the remainder as O,

(ii) methylphosphonates

(iii) phosphoramidates

(iv) phosphotriesters

(v) phosphorus-free linkages (e.g. carbamate, acetamidate, acetate,)

b) Sugar modifications:

(i) 2'-deoxynucleosides (R=H)

(ii) 2'-O-methylated nucleosides (R = OMe)

(iii) 2'-fluoro-2'-deoxynucleosides (R = F)

(iv) 2'-O-alkylated nucleosides

c) Base modifications:

(i) pyrimidine derivatives substituted in the 5-position (e.g. methyl, bromo, fluoro etc. or replacing a carboxyl group by an amino group,

(ii) purine derivatives lacking specific nitrogen atoms (e.g. 7-deaza-adenine, hypoxanthine, or functionalised in the 8-position (e.g. 8-azido adenine, 8-bromo adenine), or additional functionalities (.e.g. 2,6-diaminopurine).

d) Oligonucleotides covalently linked to reactive functional groups (e.g. psoralens, phenanthrolines, mustards).

e) irreversible cross-linking agents with or without the need for co-reagents)

(i) acridine (intercalating agents)

(ii) thio derivatives (reversible disulphide formation with proteins)

(iii) aldehydes (Schiff's base formation)

(iv) azido, bromo groups (UV cross-linking)

(v) ellipticenes (photolytic cross-linking)

f) oligonucleotides containing haptens or other binding groups;

g) fluorescent moieties or other non-radioactive labels); and

h) combination of two or more modifications selected from (a) to (g) above.

Assays

The above-defined compounds of the invention can be used in a variety of ways. Of particular interest is their use in assays, particularly screening assays. Rather than screen potential antivirals (test compounds) against virus in culture, or against artificial reporter gene constructs, they can be screened against a compound of the invention. Compounds that interact with the mIRES will typically inhibit the formation of the eIF3/5'-UTR complex *in vivo*.

Therefore the invention provides a screening assay, comprising the steps of (a) incubating a test compound with a compound of the invention, and (b) detecting the formation of a binding complex.

The assay may involve a test compound or compound of the present invention which is labelled (*e.g.* with an isotopic or non-isotopic label, such as a fluorescent label). The binding complex can be detected by monitoring changes in the signal of the label when the test compound binds to the compound of the present invention. As an alternative, the assay may involve the compound of the invention immobilised on a solid surface and a labelled test compound, with the binding complex being detected by detecting label bound to the solid surface.

According to one embodiment of the present invention there is provided a screening assay comprising the steps of (a) incubating a test compound with a compound of the present invention and with a ligand capable of binding to the compound of the present invention, and (b) determining the amount of complex between the compound of the present invention and the ligand.

The ligand and the compound of the present invention may be separate or may be in the form of a complex before addition of the test compound. The assay may measure the extent to which the test compound inhibits the formation of a complex between the compound of the present invention and the ligand or may measure the extent to which the test compound displaces the ligand from complexation with the compounds of the present invention.

Ligands useful in the present invention are capable of binding to a compound of the present invention and may be readily identified as described herein. Preferably, the ligand is a polypeptide. In one embodiment, the ligand may comprise eIF3 or a fragment thereof. Fragments of eIF3 include the individual polypeptide claims which make up eIF3, which include p170, p116, p110, p66, p48, p47, p44, p40, p37, and p35 [ref. 8, page 3186]. Thus in a one embodiment, the invention provides a screening assay, comprising the steps of (a) incubating a potential HCV antiviral with a compound of the invention and eIF3 or a fragment thereof, and (b) determining the amount of complex formed between the compound of the invention and eIF3 or a fragment thereof.

Preferably the ligand is capable of binding the mIRES.

In one embodiment the assay may be based on that described in PCT/GB99/01761, which is incorporated herein by reference. The assay employs a target molecule comprising a

compound of the present invention; and a reporter molecule comprising a ligand capable of binding to the compound of the present invention.

According to this aspect, the invention provides a method for determining whether a test compound is capable of binding to an HCV 5'-UTR, the method comprising the steps of:

(a) contacting the test compound with a pair of indicator molecules comprising (i) a reporter labelled with a donor group or an acceptor group and (ii) a compound according to the present invention labelled with a complementary acceptor or donor group, the pair being capable of binding to each other in an orientation that permits the donor group to come into sufficient proximity to the acceptor group to permit fluorescent resonance energy transfer and/or quenching to take place; and

(b) measuring the fluorescence of the compound of the present invention and/or the reporter ligand in the presence of the test compound and comparing this value to the fluorescence of a standard.

In preferred embodiments, the standard comprises:

- the indicator pair in the absence of test compound;
- the indicator pair in the presence of test compound;
- individual member(i) of the indicator pair in the absence of test compound;
- individual member (ii) of the indicator pair in the absence of test compound;
- individual member (i) of the indicator pair in the presence of test compound; and/or
- individual member (ii) of the indicator pair in the presence of test compound.

It will be appreciated that various combinations of these six choices can be used.

It will be appreciated that the fluorescence of the standard may have been determined before performing the method, or may be determined during or after the method has been performed. It may be an absolute standard.

In relation to this aspect of the present invention, the compounds of the present invention are referred to as the target.

The method may also be used in the identification of compounds that bind to the target from within a plurality of test compounds, such as in screening methods. The method may, therefore, involve the initial step of providing a plurality of test compounds.

In a typical embodiment, therefore, the invention provides a method of screening for potential antivirals, comprising the steps of (a) contacting a test compound with an indicator complex, the indicator complex comprising a fluorescently-labelled reporter ligand bound to a fluorescently labelled compound of the present invention in an orientation that permits the fluorescent groups present on each molecule to come into sufficient proximity to permit fluorescent resonance energy transfer to take place; and (b) measuring the fluorescence of the compound of the present invention and the reporter ligand in the presence of the test compound and comparing this value to the fluorescence of a standard.

The reporter ligand

The reporter ligand is capable of binding to the target and preferably forms a one-to-one complex with the target. Reporter ligands can thus be virtually any agent including, without limitation, peptides, peptoids, proteins, lipids, polysaccharides, derivatives of these, and small organic molecules with molecular weights of more than 200 and less than about 2,500 daltons, preferably between 500 and 1,000 daltons.

The reporter preferably binds the target with a K_d of between $1x10^{-12}$ and $1x10^{-4}$ M. Preferred values are lower than 500nM.

In preferred embodiments of the invention, the reporter ligand comprises a linear peptide or derivative thereof, a cyclic peptide or derivative thereof, a linear or cyclic peptoid or derivative thereof, or a peptidomimetic analogue. Linear peptides, peptoids and derivatives thereof are between 2 and 100 residues in length, preferably between 4 and 40 residues in length, and most preferably between 8 and 20 residues in length and may comprise either D- or L-amino acids (or equivalents). Cyclic peptides, peptoids and derivatives thereof are preferably between 4 and 10 residues in length, preferably 4 to 7, and may comprise either D- or L-amino acids (or equivalents). Peptoids are isomers of peptides which have side chains carried by backbone nitrogens (N-substituted glycines) (*eg.* Bartlett, *et al.* WO91/19735, Zuckermann, *et al.* WO94/06451 and Simon *et al.*, 1992). Peptoids are more flexible than peptides since intramolecular CO-HN hydrogen bonds are removed and the steric interactions that induce secondary structure are different.

Fluorescent labelling

The target and the reporter ligand may be fluorescently labelled by any suitable method, preferably by covalent attachment of a fluorescent group. The labels may be any fluorescent

label or fluorophore that does not interfere with the ability of the reporter to interact with the target and is able show quenching and/or fluorescence resonance energy transfer with the corresponding label on the target.

The target may be fluorescently labelled at any suitable position. In some embodiments, the fluorescent group or quenching group is placed on or adjacent to the 5' end of the RNA target. In other embodiments, it may be placed on or adjacent to the 5' end of one of a pair of oligonucleotides forming an RNA duplex, or the 5' end one of the component oligonucleotides in RNA structure created by the annealing of three or more RNA oligonucleotides. In other embodiments, the fluorescent group may be placed on or adjacent to the 3' end of one of the synthetic RNA molecules.

In other embodiments, the fluorescent group may be placed within the chain of the synthetic RNA molecules, for instance by incorporation of a fluorescent nucleotide derivative, modification of a nucleotide or substitution of a nucleotide by a fluorescent molecule. For example, tetramethylrhodamine (TAMRA) can be introduced into synthetic RNA by incorporating the modified deoxy-uridine phosphoramidite (5'-Dimethoxytrityloxy-5-[N-((tetramethylrhodaminyl)-aminohexyl)-3-acryimido]-2'-deoxy-uridine-3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite). Fluorescein may be incorporated in an analogous way with: 5'-Dimethoxytrityloxy-5-[N-((3',6'-dipivaloylfluoresceinyl)-aminohexyl)-3-acryimido]-2'-deoxy-uridine-3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite. The DABCYL group may also be incorporated using 5'-Dimethoxytrityloxy-5-[N-((4-(dimethylamino)azobenzene)-aminohexyl)-3-acryimido]-2'-deoxy-uridine-3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite. More generally, a free amino group may be reacted with the active ester of any dye, such an amino group may be introduced by the inclusion of the modified uridine 5'-Dimethoxytrityl-5-[N-(trifluoroacetylaminohexyl)-3-acrylimido]-2'-deoxy-uridine,3'-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite. The incorporation of a single deoxy-uridine often does not significantly perturb RNA structure and the modification at the 5 position of the base allows for normal base-pairing.

It is also possible to include more than one fluorescent label on a synthetic target molecule without departing from the scope the invention. In one embodiment of this invention, a target molecule is labelled with two fluorescent groups, for example with one group placed adjacent to the 5' end of the target RNA sequence and a second fluorescent group placed adjacent to the 3' end of the target RNA sequence. In other embodiments, two or more fluorescent groups are

placed adjacent to the 5' and/or 3' ends of the target RNA molecule and/or at internal sites in the RNA target sequences. Multiply labelled target RNAs can be used to increase the intensity of the signals detected in the assay.

In certain embodiments of the invention a target labelled at two or more positions may be used to detect interactions with two or more reporter ligands. The reporter ligands can either be used individually or simultaneously.

The reporter ligand may also be labelled at any suitable position. When peptides or peptoids are used as reporter ligands, the fluorescent group may, for instance, be placed at either the carboxyl or amino terminus of the ligand. In other embodiments using peptides or peptoids the fluorescent group may be placed on a side chain within the peptide or peptoid sequence.

It is also possible to include more than one fluorescent label on the reporter ligand without departing from the scope the invention. Multiply labelled reporter ligand can be used to enhance signal intensity and/or selectivity.

Useful fluorophores (in addition to those listed in PCT/GB99/01761) include, but are not limited to: Texas Red™ (TR), Lissamine™ rhodamine B, Oregon Green™ 488 (2',7'-difluorofluorescein), carboxyrhodol and carboxyrhodamine, Oregon Green™ 500, 6-JOE (6-carboxy-4',5'-dichloro-2',7'-dimethyoxyfluorescein), eosin F_3S (6-carboxymethylthio-2',4',5',7'-tetrabromo-4,5,7-trifluorofluorescein), Cascade Blue™ (CB), aminomethylcoumarin (AMC), pyrenes, dansyl chloride (5-dimethylaminonaphthalene-1-sulfonyl chloride) and other napththalenes, PyMPO, ITC (1-(3-isothiocyanatophenyl)-4-(5-(4-methoxyphenyl)oxazol-2-yl)pyridinium bromide).

Donor/acceptor pairing

Contact between the pair of indicator molecules may occur in solution (*eg.* a test tube, dish or well of a microtitre plate) or, alternatively, either the reporter ligand or the target molecule may be adhered to a solid support (*eg.* an affinity gel, matrix, or column) by covalent or non-covalent linkages using methods known in the art. The support bound target or reporter molecule is then mixed with a solution containing the other compound of the indicator pair.

When the reporter and target are mixed, they can form a complex which brings the donor and acceptor groups into proximity. The fluorescence of, or light emitted from, the complex formed between the reporter molecule and the target is altered by fluorescence resonance energy transfer (FRET).

The donor group may be attached to either the target or to the reporter ligand. When the donor is attached to the target, the complementary acceptor is attached to the reporter ligand: conversely, when the donor is attached to the reporter ligand, the complementary acceptor is attached to the target.

As used herein, the term "donor" refers to a fluorophore which absorbs at a first wavelength and emits at a second, longer wavelength. The term "acceptor" refers to a fluorophore, chromophore or quencher with an absorption spectrum which overlaps the donor's emission spectrum and is able to absorb some or most of the emitted energy from the donor when it is near the donor group (typically between 1-100nm). If the acceptor is a fluorophore capable of exhibiting FRET, it then re-emits at a third, still longer wavelength; if it is a chromophore or quencher, then it releases the energy absorbed from the donor without emitting a photon.

Although the acceptor's absorption spectrum overlaps the donor's emission spectrum when the two groups are in proximity, this need not be the case for the spectra of the molecules when free in solution. Acceptors thus include fluorophores, chromophores or quenchers that, following attachment to either the target molecule or to the reporter ligand, show alterations in absorption spectrum which permit the group to exhibit either FRET or quenching when placed in proximity to the donor through the binding interactions of two molecules.

The donor and acceptor groups may independently be selected from suitable fluorescent groups, chromophores and quenching groups. Preferred donors and acceptors include:

- 5-FAM (also called 5-carboxyfluorescein; also called Spiro(isobenzofuran-1(3H), 9'-(9H)xanthene)-5-carboxylic acid,3',6'-dihydroxy-3-oxo-6-carboxyfluorescein);
- 5-Hexachloro-Fluorescein ([4,7,2',4',5',7'-hexachloro-(3',6'-dipivaloylfluoresceinyl)-6-carboxylic acid]);
- 6-Hexachloro-Fluorescein ([4,7,2',4',5',7'-hexachloro-(3',6'-dipivaloylfluoresceinyl)-5-carboxylic acid]);
- 5-Tetrachloro-Fluorescein ([4,7,2',7'-tetrachloro-(3',6'-dipivaloylfluoresceinyl)-5-carboxylic acid]);
- 6-Tetrachloro-Fluorescein ([4,7,2',7'-tetrachloro-(3',6'-dipivaloylfluoresceinyl)-6-carboxylic acid]);
- 5-TAMRA (5-carboxytetramethylrhodamine; Xanthylium, 9-(2,4-dicarboxyphenyl)-3,6-bis(dimethylamino);

- 6-TAMRA (6-carboxytetramethylrhodamine; Xanthylium, 9-(2,5-dicarboxyphenyl)-3,6-bis(dimethylamino);
- EDANS (5-((2-aminoethyl)amino)naphthalene- 1-sulfonic acid) ;
- 1,5-IAEDANS (5-((((2-iodoacetyl)amino)ethyl) amino)naphthalene-1-sulfonic acid);
- DABCYL (4-((4-(dimethylamino)phenyl) azo)benzoic acid);
- Cy5 (Indodicarbocyanine-5);
- Cy3 (Indodicarbocyanine-3); and
- BODIPY^{TM} FL (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid)

as well as suitable derivatives thereof.

In preferred embodiments, the target molecule has been specifically labelled by a donor/acceptor that is different from the acceptor/donor that is present on the reporter molecule. Preferred combinations of donors and acceptors are listed, but not limited to, the donor/acceptor pairs shown in PCT/GB99/01761.

As used herein, references to "fluorescence" or "fluorescent groups" or "fluorophores" include luminescence, luminescent groups and suitable chromophores, respectively. In the present invention the target and reporter molecule may be labelled with luminescent labels and luminescence resonance energy transfer is indicative of complex formation. Suitable luminescent probes include, but are not limited to, the luminescent ions of europium and terbium introduced as lanthium chelates (Heyduk & Heyduk, 1997). The lanthanide ions are also good donors for energy transfer to fluorescent groups (Selvin 1995). Luminescent groups containing lanthanide ions can be incorporated into nucleic acids utilising an 'open cage' chelater phosphoramidite.

In certain embodiments of the invention, the target and reporter molecule may also be labelled with two chromophores, and a change in the absorption spectra of the label pair is used as a detection signal, as an alternative to measuring a change in fluorescence.

Measurable changes

In this embodiment of the present invention, the labelled reporter is capable of binding to the labelled target, thereby forming a complex in which the donor present on one molecule comes into proximity with the acceptor on the other molecule. This results in reduced fluorescence of

the complex compared to the uncomplexed fluorescence exhibited by the reporter molecule and/or target when free in solution.

In this embodiment of the invention, fluorescence intensity of the reporter molecule, the fluorescence intensity of the target and the fluorescence intensity of the complex is measured at one or more wavelengths with a fluorescence spectrophotometer or microtitre plate reader. It is generally preferred that the reporter molecule and target form a one-to-one complex and equimolar concentrations of reporter molecule and target are present in the binding reaction. However, an excess of one reagent may be used without departing from the scope of the invention.

In some embodiments, a fraction of the reporter molecules and target molecules in the binding reaction can be replaced by unlabelled analogues. The optimal proportions of labelled and unlabelled reporter and target molecules can be determined by titration of the different components and measuring the optimal concentrations required in order to obtain maximal FRET or fluorescent quenching.

The labelled target and labelled reporter molecules are then mixed with a test compound and the fluorescence in the mixture is measured. If the test compound is able to bind to the region of the target that binds to the reporter molecule, then a fraction of the reporter molecule will be prevented from binding to the target. The proportions of the free reporter, free target and complex can be quantitatively determined by comparing the spectral properties of the complex, partially dissociated complex and the uncomplexed target and reporter molecules. The amount of reporter displacement will be a function of the relative affinity of the test compound for the target compared to the reporter molecule and the relative concentrations of the two molecules in the sample. Preferably, a variety of different concentrations of the molecule to-be-tested are compared to generate a binding curve. Saturation of the target is reached when the fluorescence emission of the reporter or target molecule is restored to the levels obtained from the free molecules.

The concentration of compounds binding to targets can be determined with a fluorescence standard curve depicting the fluorescence of the labelled reporter and target with varying known concentrations of competing unlabelled test compound.

In some embodiments of the invention, fluorescence resonance energy transfer between the donor and acceptor may give rise to a distinct fluorescence emission spectrum of the complex

which can be compared to the fluorescence emission spectra of the separate reporter and target molecules.

In preferred embodiments of the invention, FRET is detected by steady state measurements of the integrated emission intensity of the donor (*ie.* the fluorescent dye that is excited by the light source used in the spectral measurement) and/or the acceptor (*ie.* the fluorescent dye which has an absorption spectrum that overlaps the emission spectrum of the donor). In other embodiments of the invention FRET may be detected by time-resolved measurements in which the decay of donor fluorescence is measured after a short pulse of excitation. In certain embodiments of the invention the donor is excited at a wavelength that does not itself result in efficient excitation of the acceptor, and FRET is detected by measuring the excitation of the acceptor due to transfer of a photon from the donor.

The transfer of energy from donor to acceptor is associated with a reduction of the intensity of the fluorescence exhibited by the donor (quenching). In certain preferred embodiments of the invention, only quenching of the donor due to the proximity of the acceptor in the reporter/target complex is measured. In certain embodiments of the invention, the target carries a chromophore or fluorophore that quenches the fluorescence of the fluorescent group on the reporter after binding of the two molecules. In other embodiments of the invention the reporter carries a chromophore or fluorophore that quenches the fluorescence of the fluorescent group on the target after binding of the two molecules.

It will typically be preferable to look for a signal (a positive), rather than for the absence of a signal (a negative), but it will be appreciated that either or both may be followed.

Order of mixing

In this embodiment of the invention it is preferred that the target, the reporter, and the test compound are mixed, and the fluorescence of the mixture is compared to standards. Competitive inhibitors of the binding of the reporter molecule prevent the formation of the reporter-target complex and therefore increase the amount of free target and free reporter in the reaction. Since the fluorescence of the free and reporter molecules is unquenched, the overall fluorescence in the reaction increases in direct relation to the amount of test compound in the binding reaction and its relative affinity for the target compared to the reporter molecule.

Alternatively, the test compound is first mixed with the labelled target in order to form a complex in the absence of the labelled reporter, and the reporter is then added. Since the reporter molecule will only be able to bind to the free target in the reaction, there will be a reduced amount of complex formed between the reporter and the target compared to the amount of complex formed in the absence of test compound. As a result, the fluorescence of the mixture containing the test compound will be increased compared to a similar mixture prepared in the absence of the test compound.

In another alternative, a complex is pre-formed between the labelled target and the labelled reporter molecule before addition of the test compound. If the test compound is able to disrupt the complex formed between the labelled-target and the labelled-reporter molecule, or alter the equilibrium binding state by binding to target that has dissociated from the reporter molecule, the amount of complex in the reaction will be reduced and the overall fluorescence of the mixture will increase.

In some circumstances the test compound may itself be fluorescent and/or be capable of quenching the fluorescent group present on the target and/or the reporter molecule. Preferably, the fluorescence of standards containing the test compound on its own, and in pairwise combinations with the target or reporter molecules, are measured and these values are compared to the fluorescence of the complete test mixture containing the test compound, the fluorescent target and the reporter molecule.

Quenching of fluorescence arising from the target due to the binding of the test compound to the target will result in a decrease in the signal arising from the target that is not complexed to the reporter molecule, but will not affect the fluorescent signal arising from the group on the reporter molecule or the signal obtained from the target in a complex with the reporter molecule. In this circumstance it is preferable to configure the donor/acceptor pairs on the target and the reporter molecule such that an increase in the fluorescence of the reporter molecule is detectable when the formation of the complex between the reporter and the target is blocked by the test compound.

Library screening (including high throughput screens)

The present invention also encompasses high-throughput screening methods for identifying compounds that bind to the target. Preferably, all the biochemical steps for this assay are performed in a single solution in, for instance, a test tube or microtitre plate, and the test compounds are analysed initially at a single compound concentration. For the purposes of high

throughput screening, the experimental conditions are adjusted to achieve a proportion of test compounds identified as "positive" compounds from amongst the total compounds screened. The assay is preferably set to identify compounds with an appreciable affinity towards the target *eg.* when 0.1% to 1% of the total test compounds from a large compound library are shown to bind to a given target with a K_i of 10 μM or less (*eg.* 1 μM, 100 nM, 10 nM, or less).

Reporter identification

The methods of the invention require a suitable reporter molecule. Accordingly, another aspect of the invention is a method for identifying a reporter molecule from a mixture (*eg.* a combinatorial library) of labelled peptides, peptoids or other polymers carrying side chains.

In one example of such a method, a series of peptides or peptoids between 3 and 100 residues in length are synthesised with a mixed collection of side chains (containing either natural amino acid side chains or sequence variants) at several positions within the sequence (*eg.* Felder *et al.*, WO96/40759, and Hamy *et al.*, 1997) and a fluorescent moiety (either a donor or an acceptor) is placed at either the C-terminal or N-terminal end.

The individual compounds are then mixed in solution with the target of interest that has been labelled with a complementary donor or acceptor. Complexes between the individual test compound and the target are detected by measuring quenching and/or fluorescence resonance energy transfer. Preferably, the compounds are tested over a range of different concentrations (between 10 nM and 1 mM) and the target is at a fixed concentration (between 10 nM and 100 nM). The compounds are then ranked by calculating K_d for each compound and the target RNA pair. The compound with the lowest K_d is then selected for use as a reporter.

Kits of the invention

The invention also provides a kit for determining whether a test compound binds to a compound of the present invention, the kit comprising (a) a compound of the present invention and (b) a ligand capable of binding the compound of the present invention, wherein either or both the ligand and the compound of the present invention are labelled.

In a preferred embodiment provides a kit for determining whether a test compound is capable of binding to an HCV 5'-UTR, the kit comprising (a) a compound of the present invention labelled with a donor group or an acceptor group and (b) a reporter labelled with a complementary acceptor or donor group, wherein the reporter and the compound of the present invention are capable of binding to each other in an orientation that permits the donor

group to come into sufficient proximity to the acceptor group to permit fluorescent resonance energy transfer and/or quenching.

The invention also provides an HCV antiviral identified by such a screening assay and use of such an antiviral in the treatment of HCV.

There is also provided a method for formulating an HCV antiviral, comprising the steps of (a) performing a screening assay according to the invention, (b) selecting an antiviral that inhibits the formation of an eIF3/5-UTR mimic complex, and (c) formulating the antiviral as a pharmaceutical.

The invention further provides the use of the compounds of the invention in testing potential HCV antivirals.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows the 5'-UTR of HCV strain 1a (M67463). The boxed region is the mIRES of the invention.

Figure 2 shows the mIRES in generalised structural formula.

Figure 3 is an alignment of the two short sequences that make up the mIRES from published HCV genomic sequences (shown in DNA form, with T instead of U). These 35 pairs of sequences are those which were represented more than once in an alignment of more than 600 wild-type HCV sequences.

Figure 4 illustrates the mIRES of Figure 1 in a heterologous context, flanked by at least five Watson-Crick base pairs on each side.

Figure 5 shows HCV 5'-UTR Domain III EMSA RNA probes.

Figure 6 shows (a) a fluorescein labelled RNA construct containing the mIRES and (b) a 19-mer RNA control construct.

Figure 7 shows binding cures for the RNA construct containing the mIRES titrated with various peptides.

Figure 8A shows paramomycin-TAMRA binding to an HCV-derived RNA corresponding to mIRES IIIb.

Figure 8B shows competition of the binding reaction of Figure 8A with neomycin.

MODES FOR CARRYING OUT THE INVENTION

A. Identification of the mIRES

The effects of mutations in the IIIb loop in HCV were determined by IRES-dependent translation assay.

A striking reduction in IRES-dependent translation was noted when C at nucleotide 186 was changed to G, thereby creating a W•C base pair with nucleotide 211. Translation was only 39% of that driven by the wild-type sequence. Similarly, replacement of nucleotides 182 and 183 (AC) with CAUU, to form W•C base pairs with nucleotides 214 to 217, reduced translation to 24% of wild-type. Replacement of nucleotides 214 to 217 (AAUG) with GU, to form W•C base pairs with nucleotides 182 and 183, reduced translation to 34% of wild-type.

The two bulged regions boxed in Figure 1 are thus critical for IRES-driven translation. It is concluded that the binding interaction between eIF3 and the 5'-UTR in HCV genotype 1a requires the motif formed by the annealing of two short linear sequences 5'-G-A-C-G-A-C-C-3' and 3'-C-G-U-A-A-C-U-C-G-5'. These anneal to form the mIRES.

It is surprising that two such short sequences are essential to the binding of eIF3 and can be used to mimic the full-length 5'-UTR. Firstly, interest has focused on domains IIIa and IIIc [4], although some interactions with IIIb have been noted in crude footprinting studies [6]. Secondly, the two short linear sequences are by no means specific for HCV. The 7mer sequence GACGACC is found in, for instance, human chromosome 19 (accession AC005602), pig Na-dependent glucose cotransporter (L02900), rat Id3a (AF000942, D10864), mouse helix-loop-helix protein (M60523), *C.elegans* collagen (Z22964), *Halobacter* NRC-1 plasmid (AF016485), and vWF from *Bradypus tridactylus* (U31603) and *Chaetophractus villosus* (AF076480). The 9mer sequence GCUCAAUGC is found in *Xenopus laevis* corticotropin releasing factor receptor (Y14036), human MHC1 (AF055066), *Synechocystis* PCC6803 (D90910), and mouse tenascin (D90343). Identity of the two mIRES constituent sequences from other HCV strains with non-HCV sequences is similarly prevalent. The two sequences individually, therefore, are not specific to HCV, but their juxtaposition in a three-dimensional annealed structure gives rise to an effective mimic of the native 5'-UTR.

B. Mutation analysis of the mIRES

To probe the critical residues within these 2 short regions, the mIRES was dissected internally.

The following single nucleotide mutations were made:

Nucleotide	Wild-type residue	Mutation	Translation
182	A	C	68%
		G	56%
		U	76%
183	C	A	66%
		G	63%
		U	80%
184	G	A	30%
		U	30%
185	A	U	39%
		G	26%
		C	43%
186	C	G	39%
187	C	G	39%
211	C	A	31%
		G	21%
		U	55%
212	U	A	52%
		G	44%
		C	53%
213	C	A	44%
		G	39%
		U	78%
214	A	C	92%
		G	73%
		U	90%
215	A	G	93%
		U	92%
216	U	C	72%
		G	84%
217	G	A	100%
		C	55%
		U	88%

Pairs of mutations were also tested. The collected results were as follows:

Nucleotide pair	Wild-type residues	Mutation	Translation
182-217	A-G	C-G	68%
		G-G	56%
		U-G	76%
		A-A	100%
		A-C	55%
		A-U	88%
		C-A	66%
		C-C	33%
		C-U	43%
		G-A	97%
		G-C	72%
		G-U	89%
		U-A	44%
		U-C	61%
		U-U	63%
183-214	C-A	A-A	66%
		G-A	63%
		U-A	80%
		C-C	92%
		C-G	73%
		C-U	90%
		A-C	79%
		A-G	40%
		A-U	89%
		G-C	91%
		G-G	74%
		G-U	90%
		U-G	86%
		U-U	78%
184-213	G-C	A-C	30%
		U-C	30%
		G-A	44%
		G-G	39%
		G-U	78%
185-212	A-U	U-U	39%
		G-U	26%
		C-U	43%
		A-A	52%
		A-G	44%
		A-C	53%
		U-A	90%
		U-G	43%
		U-C	45%
		G-A	49%
		G-G	27%
		G-C	34%
		C-A	54%
		C-G	71%
		C-C	45%
186-211	C-C	G-C	39%
		C-A	31%
		C-G	21%
		C-U	55%
		G-G	35%
		G-U	30%

One triple mutant was also tested ($C^{183}\rightarrow A + A^{214}\rightarrow G$ + insertion of U at nucleotide 211). This reduced translation to 8% of wild-type.

From the above data, it is concluded that the mIRES will tolerate the following mutations whilst remaining functional:

Nucleotide	Wild-type	Functional mutations
182	A	Can be mutated to C, G or U, provided that when it is C, nucleotide 217 is not also C
183	C	Can be mutated to U, G or A, provided that when it is A, nucleotide 214 is not G
184	G	None
185	A	Can be mutated to C, G or U, provided that (a) nucleotide 212 is also mutated, and (b) when it is G, nucleotide 212 is A.
186	C	None.
211	C	Can be mutated to U.
212	U	Can be mutated to A, C or G, provided that when it is C or G, nucleotide 185 is not G.
213	C	Can be mutated to A, U or G.
214	A	Can be mutated to U, C or G, provided that, when it is G, nucleotide 183 is not A.
215	A	Can be mutated to G or U or C
216	U	Can be mutated to C or G or A
217	G	Can be mutated to A, C or G, provided that when it is C, residue 182 is not also C.

C. Analysis of the interaction between eIF3 and the HCV IRES

Methods

The ability of eIF3 to interact with the HCV IRES was assessed with a RNA electrophoretic gel mobility shift assay (ESMA). The eIF3 complex was studied with the complete IRES RNA, Domain III and deletions of Domain III. The ability of each probe to form a complex with eIF3 was determined by EMSA. In addition, to determine the relative affinity of each IRES RNA competition analysis was performed with unlabelled RNA.

Preparation of radiolabelled IRES probes and unlabelled competitors

Plasmid templates (Fig. 5), linearised with EcoRI, were transcribed with T7 RNA polymerase in the presence of [32p]UTPor UTP and purified by denaturing electrophoresis and subsequent

electroelution. The concentration of each prepared RNA was determined by UV spectroscopy and confirmed by denaturing electrophoresis.

EMSA Binding assay

IRES-eIF3 binding reactions were performed by incubating the radiolabelled probe (1nM) with 0.01 µg of purified on ice, in a total volume of 18 µl of binding buffer (60 mM KCl, 10 mM Hepes pH 7.4, 3.0 mM $MgCl^2$, 1 mM DTT, 5% glycerol and 200 nM tRNA). The complex was incubated on ice for 10 min prior to electrophoresis. For competition experiments, unlabelled RNA was pre-incubated with eIF3 for 10 min prior to the addition of radiolabelled probe. The eIF3-IRES complex was resolved by non-denaturing electrophoresis. The gels were dried and quantified by phosphorimager analysis.

Results

EMSA analysis clearly indicates that both the complete IRES and the isolated Domain III form a specific interaction with eIF3. Deletions of Domain III, DIIIΔ1 and DIIIΔ2, also form a specific complex with eIF3, albeit with reduced affinity. Further deletions of Domain III abolish complex formation. In addition, other regions of the IRES, such as Domain II both fail to bind eIF3 and demonstrate significant competition. This is also true for tRNA, an unrelated RNA. Deletions of Domain III with a relative affinity less than 4% (compared to Domain III) do not demonstrate selectivity (Table 1).

In summary, although the mIRES is critical for IRES activity and the interaction with eIF3, the mIRES is not sufficient for eIF3 binding. Other determinants of Domain III, such as domain IIIa/c are required for the interaction. In addition, the eIF3 EMSA system provides an assay to quantify the inhibitory activity of HCV IRES therapeutic compounds

RNA	Ability to complex with eIF3	Relative binding affinity
IRES (complete)	+	N/D
DIII	+	100%
DIIIΔ1	+	20%
DIIIΔ2	+	4%
DIIIΔ3	-	1%
DII	-	0.5%
tRNA	-	0.1%

Table 1. Binding properties of Domain III probes with eIF3

D. FRET ASSAY FOR THE HCV INTERNAL LOOP (mIRES)

Library of RNA-binding peptides

To identify peptides binding to HCV IRES RNA, a library of peptides with a variable pentapeptide core and constant flanking neutral and basic amino acids was designed. All peptides contained the acceptor dye Dabsyl at the N-terminus. The three amino acids K, Q and R were used in the variable pentapetide part. From the possible 243 (3^5) peptides a subset of 40 peptides was chosen for synthesis and testing (Table 2).

Table 2: Library of RNA-binding peptides. The variable core is highlighted.

No.																
3	N-Dabsyl	T	R	K	K	K	K	K	K	R	K	G	S	G	-Amide
4	N-Dabsyl	T	R	K	K	K	K	Q	R	R	K	G	S	G	-Amide
5	N-Dabsyl	T	R	K	K	K	Q	K	R	R	K	G	S	G	-Amide
6	N-Dabsyl	T	R	K	K	K	Q	R	R	R	K	G	S	G	-Amide
7	N-Dabsyl	T	R	K	K	K	R	Q	R	R	K	G	S	G	-Amide
8	N-Dabsyl	T	R	K	K	Q	K	K	R	R	K	G	S	G	-Amide
9	N-Dabsyl	T	R	K	K	Q	K	R	R	R	K	G	S	G	-Amide
10	N-Dabsyl	T	R	K	K	Q	Q	Q	R	R	K	G	S	G	-Amide
11	N-Dabsyl	T	R	K	K	Q	R	K	R	R	K	G	S	G	-Amide
12	N-Dabsyl	T	R	K	K	Q	R	R	R	R	K	G	S	G	-Amide
13	N-Dabsyl	T	R	K	K	R	K	Q	R	R	K	G	S	G	-Amide
14	N-Dabsyl	T	R	K	K	R	Q	K	R	R	K	G	S	G	-Amide
15	N-Dabsyl	T	R	K	K	R	Q	R	R	R	K	G	S	G	-Amide
16	N-Dabsyl	T	R	K	K	R	R	Q	R	R	K	G	S	G	-Amide
17	N-Dabsyl	T	R	K	Q	K	K	K	R	R	K	G	S	G	-Amide
18	N-Dabsyl	T	R	K	Q	K	K	R	R	R	K	G	S	G	-Amide
19	N-Dabsyl	T	R	K	Q	K	Q	Q	R	R	K	G	S	G	-Amide
20	N-Dabsyl	T	R	K	Q	K	R	K	R	R	K	G	S	G	-Amide
21	N-Dabsyl	T	R	K	Q	K	R	R	R	R	K	G	S	G	-Amide
22	N-Dabsyl	T	R	K	Q	Q	K	Q	R	R	K	G	S	G	-Amide
23	N-Dabsyl	T	R	K	Q	Q	Q	K	R	R	K	G	S	G	-Amide
24	N-Dabsyl	T	R	K	Q	Q	Q	Q	Q	R	K	G	S	G	-Amide
25	N-Dabsyl	T	R	K	Q	Q	Q	R	R	R	K	G	S	G	-Amide
26	N-Dabsyl	T	R	K	Q	Q	R	Q	R	R	K	G	S	G	-Amide
27	N-Dabsyl	T	R	K	Q	R	K	Q	Q	R	K	G	S	G	-Amide
28	N-Dabsyl	T	R	K	Q	R	K	K	R	R	K	G	S	G	-Amide
29	N-Dabsyl	T	R	K	Q	R	K	R	R	R	K	G	S	G	-Amide
30	N-Dabsyl	T	R	K	Q	R	Q	Q	R	R	K	G	S	G	-Amide
31	N-Dabsyl	T	R	K	Q	R	R	K	R	R	K	G	S	G	-Amide
32	N-Dabsyl	T	R	K	Q	R	R	R	R	R	K	G	S	G	-Amide
33	N-Dabsyl	T	R	K	R	Q	K	K	R	R	K	G	S	G	-Amide
34	N-Dabsyl	T	R	K	R	Q	K	R	R	R	K	G	S	G	-Amide
35	N-Dabsyl	T	R	K	R	Q	Q	Q	R	R	K	G	S	G	-Amide
36	N-Dabsyl	T	R	K	R	Q	R	K	R	R	K	G	S	G	-Amide
37	N-Dabsyl	T	R	K	R	Q	R	R	R	R	K	G	S	G	-Amide
38	N-Dabsyl	T	R	K	R	R	K	Q	R	R	K	G	S	G	-Amide
39	N-Dabsyl	T	R	K	R	R	Q	K	R	R	K	G	S	G	-Amide
40	N-Dabsyl	T	R	K	R	R	Q	R	R	R	K	G	S	G	-Amide
41	N-Dabsyl	T	R	K	R	R	R	Q	R	R	K	G	S	G	-Amide
42	N-Dabsyl	T	R	K	R	R	R	R	R	R	K	G	S	G	-Amide

Fluorescein-labelled RNAs

Oligoribonucleotides with a fluorescein attached to the 5' end of the molecule (shown in Figure 6a) were synthesised using conventional oligoribonucleotide techniques. A 19mer (Figure 6b) was used for control purposes.

Measurement of RNA/peptide interaction by FRET

Fluorescein-labelled RNA was titrated with Dabsyl-labelled peptides. The donor (fluorescein) fluorescence was excited at 490nm. In the presence of peptide, binding leads to a quenching of fluorescence by the non-fluorescent acceptor dye Dabsyl. The quenching curves allow the determination of binding affinities. Fluorescein-labelled RNA containing the mIRES was titrated with various peptides.

Figure 7 illustrates binding curves for RNA containing the mIRES titrated with various peptides. Experimental conditions: Fam-labelled RNA (10nM) was titrated with Dabsyl-labelled peptide in 50 mM Tris/Cl, pH7.4, 110 mM KCl, 5ug/ml BSA, 0.01% Triton X-100, 1%DMSO. Fluorescence (excitation 490nm/emission at 535nm) was determined in 96-well plates using a Fluorescence plate reader. The assay volume was 100µl.

Binding data for various peptides to HCV mIRES RNA.

Dissociation constants were determined by fitting a binding isotherm (1-site-binding) to the titration data using the equation

$$Y=((Fmax-Fmin)-((Fmax-Fmin)*c/(Kd+c)))+Fmin$$

wherein Fmax: fluorescence in the absence of acceptor
Fmin: fluorescence at saturation
c: peptide conc.

The FRET based displacement assay of the invention with a nM peptide ligand for the HCV mIRES identified herein provides a primary screen for compounds targeted to the internal loop that can disrupt the eIF3-5`-UTR interaction.

RNA	Peptide	Kd(nM)	R^2
HCV loopIII	RRRRR	30±10	0.96
	KKQRR	41	0.91
	KKKKK	49	0.98
	KKKQR	90	0.93
	KKRQR	104	0.95
	KKQKR	>500	0.99
	QQQQQ	>500	N/A
19mer control RNA	RRRRR	194	0.97
	KKRQR	>500	N/A
	KKQKR	>500	N/A
	KKKKK	>500	N/A
	KKQRR	>500	N/A
	KKKQR	>500	N/A
	QQQQQ	>500	N/A

Table 3: Affinities of various peptides for HCV mIRES RNA.

E. Binding of Paromomycin-TAMRA to HCV IIIB by FRET

Paromomycin-TAMRA (tetramethyl rhodamine) binding to an HCV derived RNA fragment corresponding to IIIB-Dabcyl RNA was demonstrated by titrating IIIB RNA in the presence of 10nM paromomycin as shown in Figure 8A. When Paromomycin-TAMRA binds to IIIB-Dabcyl RNA, fluorescence resonance energy transfer can take place between the fluorescent donor (TAMRA) and the non-fluorescent acceptor (dabcyl). When FRET takes place, it is observed as a reduction in fluorescent donor emission, and a concomitant increase in the fluorescent acceptor emission. The dabcyl group is not fluorescent. In this experiment FRET is measured by the reduction in donor (TAMRA) fluorescence. The reduction in TAMRA fluorescence in the presence of increasing amounts of RNA is consistent with FRET taking place between the TAMRA and dabcyl groups. This is consistent with paromomycin-TAMRA binding to IIIB RNA, the data for this experiment correspond to a Kd of 9.3 (+/- 1.5) nM for the binding of paromomycin-TAMRA to the IIIB RNA.

Inhibition of paromomycin-TAMRA binding to IIIB-Dabcyl RNA by Neomycin.

Neomycin (10uM) inhibits paromomycin-TAMRA binding to IIIB-Dabcyl RNA. Inhibition of Paromomycin-TAMRA binding to IIIB-Dabcyl RNA was demonstrated by titrating IIIB RNA with 10nM paromomycin-TAMRA in the presence of 10uM neomycin. The results are shown in Figure 8B. In this experiment FRET between the paromomycin-TAMRA and the IIIBDabcyl-RNA does not take place so there is no reduction in TAMRA fluorescence in the presence of RNA. The data are consistent with a Ki for neomycin inhibition of paromomycin-TAMRA- IIIB binding of 520 (+/- 33) nM.

Synthesis of Paramomycin-TAMRA

Paramomycin-TAMRA was synthesised by reacting 55mg paramomycin sulphate in sodium bicarbonate (6mL 0.067M in 30% dimethyl formamide (DMF) with 5mg 5-carboxytetramethyl rhodamine (in 1mL DMF) over 12 hours at room temp. The solution was diluted and purified by anion exchange chromatography, and reversed phase HPLC (ref 9).

Methods

The interaction between paromomycin-TAMRA and IIIB-Dabcyl RNA was measured utilising paromomycin-TAMRA as a donor and IIIB-Dabcyl RNA as an acceptor. The results are shown in Figure 8A. Each measurement was made in a 2mL cuvette, increasing amounts of IIIB-Dabcyl RNA (corresponding to the amounts shown in the figure) were added to a solution of 10nM paromomycin-TAMRA in the presence of 50mM Tris.HCl pH7.5, 80mM KCl. For each titration point emission spectra were acquired using a fixed wavelength of 552nm with the excitation slits set to 5nm and the emission slits set to 10nm. Emission spectra were acquired over the range 570-600nm. This range encompasses the emission spectrum of the donor (tetramethyl rhodamine). A reduction in donor intensity was observed, this is due to FRET taking place between the two dyes upon peptide binding to the IIIB- RNA. the Kd for binding was 9.3 (+/-1.5) nM. The donor ratio presented is the difference in donor intensity on addition of IIIB-RNA as a proportion of the total donor intensity in the absence of acceptor.

In Figure 8B, measurements were made in a 2mL cuvette, increasing amounts of IIIB-Dabcyl RNA (corresponding to the amounts shown in the figure) were added to a solution of 10nM paromomycin-TAMRA in the presence of 50mM Tris.HCl pH7.5, 80mM KCl. For each titration point emission spectra were acquired using a fixed wavelength of 552nm with the excitation slits set to 5nm and the emission slits set to 10nm. Emission spectra were acquired over the range 570-600nm. Two binding curves are presented, the first in the absence of compound and the second in the presence of 10μM neomycin. The presence of neomycin prevents binding and the reduction in donor signal due to FRET does not occur. Neomycin inhibits paromomycin-TAMRA to IIIB RNA binding with a Ki of 520 (+/- 33) nM.

Sequence of the RNA

RNA was synthesised by solid phase methods, a 3' Dabcyl group was incorporated as a non-fluorescent acceptor.

5'- GGG ACG ACC GCU UCG GCG CUC AAU GCC C- Dabcyl 3'

All publications mentioned in the specification are herein incorporated by reference.

Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

REFERENCES (the contents of which are incorporated herein in full)

1 Das *et al.* (1998) *Frontiers in Bioscience* 3:d1241-1252.

2 Das *et al.* (1998) *J. Virol.* 72:5638-5647.

3 Hanecak *et al.* (1996) *J. Virol.* 70:5203-5212.

4 Tang *et al.* (1999) *J. Virol.* 73:2359-2364

5 Collier *et al.* (1998) *J. Virol.* 79:2359-2366.

6 Sizova *et al.* (1998) *J. Virol.* 72:4775-4782.

7 Kieft *et al.* (1999) *J. Mol. Biol.* 292(3):513-529.

8 Buratti *et al.* (1998) *Nucleic Acid Research* 26(13):3179-3187.

9 Biochemistry 1997 36 768-779

CLAIMS:

1. A compound comprising nucleotide sequences:-

5'-P-N^1-N^2-G-N^3-C-I-3' and 3'-Q-N^4-N^5-N^6-N^7-N^8-N^9-Y-J-5'

wherein

- P and Q are any two nucleotides that can form a Watson-Crick base pair,
- I and J are any two nucleotides that can form a base pair,
- N^1 and N^4 are not both C,
- when N^2 is A, N^7 is not G,
- N^9 can only be U if N^3 is A,
- when N^3 is G, N^9 is A,

and wherein said sequences are capable of annealing, characterised in that said compound comprises 200 or fewer nucleotides.

2. A compound comprising nucleotide sequences:-

5'-P-N^1-N^2-G-N^3-C-I-3' and 3'-Q-N^4-N^5-N^6-N^7-N^8-N^9-Y-J-5'

wherein

- P and Q are any two nucleotides that can form a Watson-Crick base pair,
- I and J are any two nucleotides that can form a base pair,
- N^1 and N^4 are not both C,
- when N^2 is A, N^7 is not G,
- N^9 can only be U if N^3 is A,
- when N^3 is G, N^9 is A,

and wherein said sequences are capable of annealing, provided that said compound is not a complete HCV genome or fragment thereof.

3. A compound according to claim 1 or 2 wherein the 3' end of the sequence 5'-P-N^1-N^2-G-N^3-C-I-3' and the 5' end of the sequence 3'-Q-N^4-N^5-N^6-N^7-N^8-N^9-Y-J-5' are joined by a linker which allows said sequences to anneal.

4. A compound according to claim 3 comprising the sequence

$5'$-$(N)_a$-[P-N^1-N^2-G-N^3-C-I]-$(N)_b$-[J-Y-N^9-N^8-N^7-N^6-N^5-N^4-Q]-$(N)_c$-$3'$

wherein N may be the same or different and are any nucleotide

a is zero or an integer from 1 to 100

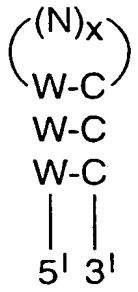
b is an integer from 3 to 100

c is zero or an integer from 1 to 100

5. A compound according to claim 4 wherein linker -$(N)_b$- is a stabilising sequence.

6. A compound according to claim 4 or 5 wherein said linker -$(N)_b$- comprises a loop.

7. A compound according to claim 6 wherein said linker -$(N)_b$- comprises the structure:



wherein x is an integer from 4 to 20.

8. A compound comprising a first nucleic acid strand comprising the sequence $5'$-P-N^1-N^2-G-N^3-C-I-$3'$ annealed to a second nucleic acid strand comprising the sequence $3'$-Q-N^4-N^5-N^6-N^7-N^8-N^9-Y-J-$5'$, wherein

- P and Q are any two nucleotides that can form a Watson-Crick base pair,
- I and J are any two nucleotides that can form a base pair, preferably a Watson-Crick base pair,
- N^1 and N^4 are not both C,
- when N^2 is A, N^7 is not G,
- N^9 can only be U if N^3 is A, and
- when N^3 is G, N^9 is A.

9. A compound according to claim 8 comprising a first nucleic acid strand comprising the sequence $5'(N)_a$-P-N^1-N^2-G-N^3-C-I-$(N)_{b^1}$-$3'$ annealed to a second nucleic acid strand comprising the sequence $3'$-$(N)_c$-Q-N^4-N^5-N^6-N^7-N^8-N^9-Y-J-$(N)_{b^2}$-$5'$, wherein

a is zero or an integer from 1 to 100,

b^1 is zero or an integer from 1 to 100,

b^2 is zero or an integer from 1 to 100, and

c is zero or an integer from 1 to 100.

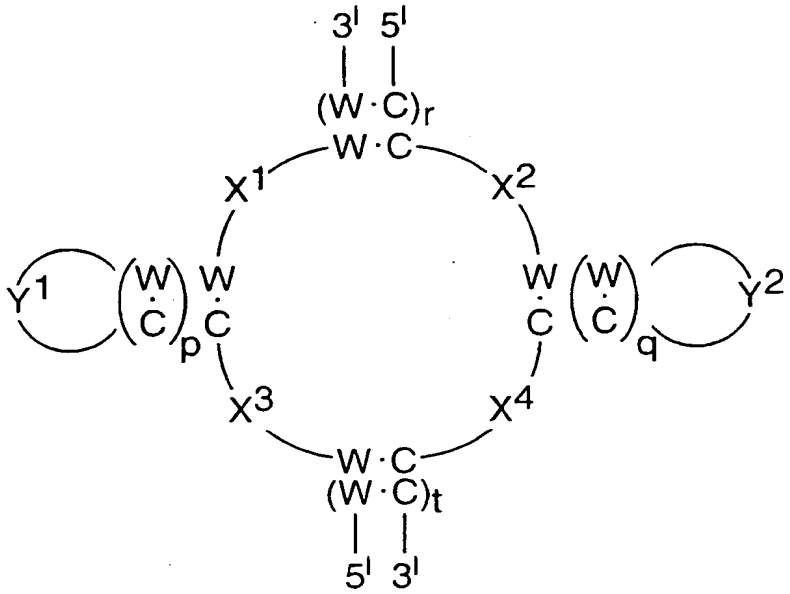
10. A compound according to claim 9 wherein $(N)_{b1}$ and $(N)_{b2}$ comprise a stabilising group.

11. A compound according to claim 9 or 10 wherein $-(N)_{b1}-$ and $(N)_{b2}$ are capable of annealing.

12. A compound according to any one of claims 4 to 7 and 9 to 11 wherein $-(N)_a-$ and $-(N)_c-$ comprise a stabilising group.

13. A compound according to any one of claims 4 to 7 and 9 to 12 wherein $-(N)_a-$ and $-(N)_c-$ are capable of annealing.

14. A compound according to claim 13 wherein $-(N)_a-$ and $-(N)_c-$ are capable of forming a structure comprising:



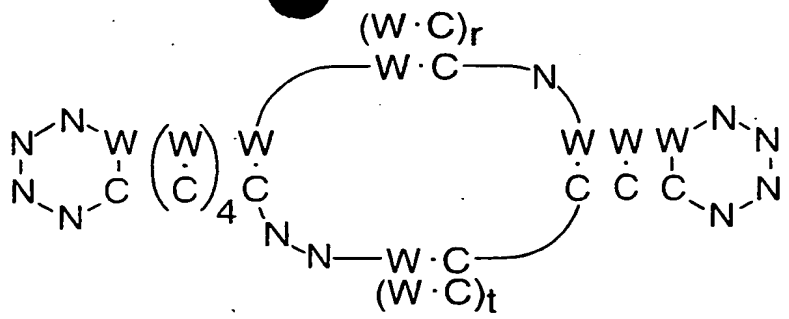
wherein each W•C may be the same or different and is a Watson-Crick base pair,

X^1, X^2, X^3 and X^4 may be the same or different and comprise from zero to four nucleotides,

Y^1 and Y^2 may be the same or different and comprise from three to ten nucleotides,

p, q, r and t may be the same or different and integers from zero to ten.

15. A compound according to claim 14 wherein $-(N)_a-$ and $-(N)_c-$ are capable of forming a structure comprising:



16. A compound according to any preceding claim wherein P is a purine; Q is a pyrimidine; I is a pyrimidine; J is a purine; N^1 is A, G or U; N^2 is A, C or U; N^3 is A; N^4 is a purine; N^5 is U; N^6 is a purine; N^7 is A, G or U; N^8 is C; N^9 is U; and Y is C.

17. A compound according to any preceding claim containing modified nucleotide bases and/or sugars and/or linkages.

18. An assay, comprising the steps of (a) incubating a test compound with a compound according to any one of claims 1 to 17, and (b) detecting the formation of a binding complex.

19. An assay according to claim 18 comprising the steps of (a) incubating a test compound with a compound according to any one of claims 1 to 17 and with a ligand capable of binding to the compound according to any one of claims 1 to 17, and (b) determining the amount of complex between compound according to any one of claims 1 to 17 and the ligand.

20. An assay according to claim 19 comprising the steps of: (a) contacting the test compound with a pair of indicator molecules comprising (i) a reporter labelled with a donor group or an acceptor group and (ii) a compound according to any one of claims 1 to 17 labelled with a complementary acceptor or donor group, the pair being capable of binding to each other in an orientation that permits the donor group to come into sufficient proximity to the acceptor group to permit fluorescent resonance energy transfer and/or quenching to take place; and (b) measuring the fluorescence of the compound according to any one of claims 1 to 17 and/or the reporter ligand in the presence of the test compound and comparing this value to the fluorescence of a standard.

21. An assay according to any one of claims 18 to 20 wherein the assay is a screening assay for potential HCV antiviral compounds.

22. A kit for determining whether a test compound binds to a compound of any one of claims 1 to 17, the kit comprising (a) a compound of any one of claims 1 to 17 and (b) a ligand capable of binding to the compound according to any one of claims 1 to 17, wherein either or both the ligand and the compound according to any one of claims 1 to 17 are labelled.

23. A kit according to claim 22 comprising (a) a compound according to any one of claims 1 to 17 labelled with a donor group or an acceptor group and (b) a reporter labelled with a complementary acceptor or donor group, wherein the reporter and the compound according to any one of claims 1 to 17 are capable of binding to each other in an orientation that permits the donor group to come into sufficient proximity to the acceptor group to permit fluorescent resonance energy transfer and/or quenching.

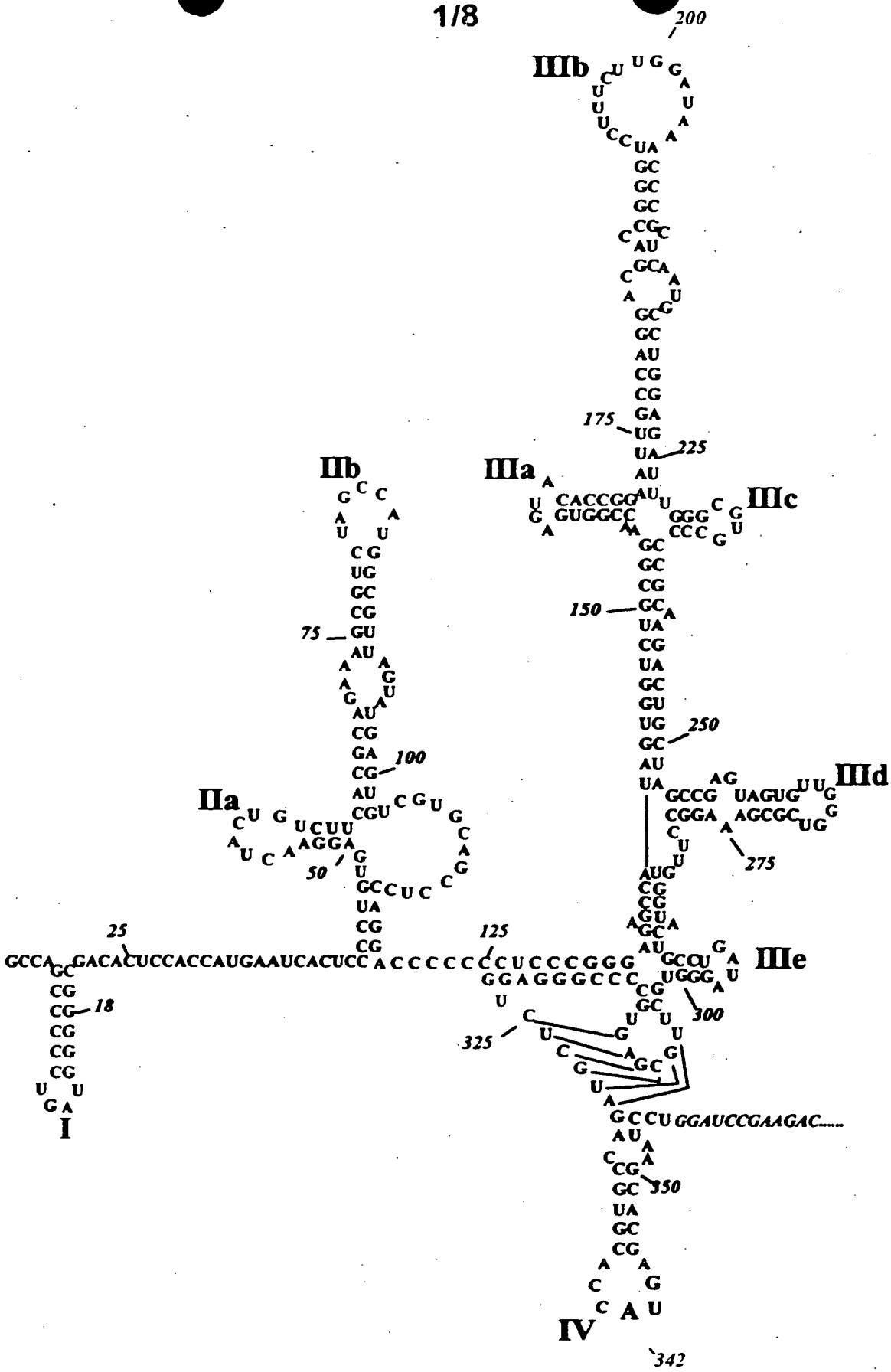

IIIb
200
175
225
IIb
IIIa
IIIc
150
75
100
IIa
250
IIId
275
50
25
125
IIIe
18
300
325
I
GCCA GACACUCCACCATGAATCACTCC
GCCU GGAUCCGAAGAC
350
IV
342

FIG. 2

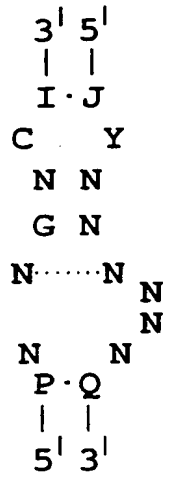


FIG. 4

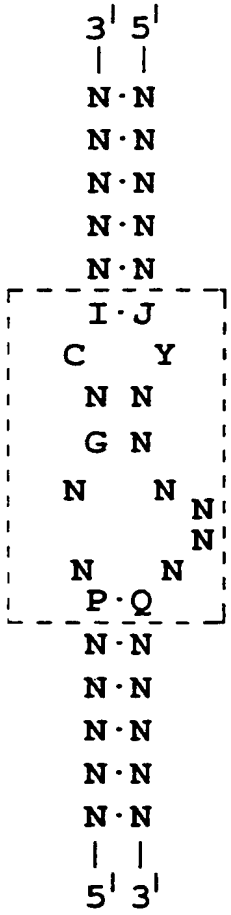
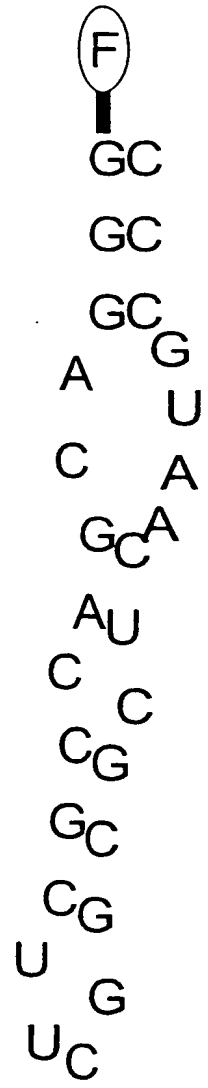


FIG. 6A



HCV mIRES RNA

FIG. 6B

19mer (control)

FIG. 3A

```
gi|1009264   GACGACC
gi|1160327   -------
gi|1438429   -------
gi|1181831   -------
gi|1197128   --A---T
gi|1805843   -GT----
gi|1930117   -GT----
gi|1066559   --A---T
gi|1066561   --T----
gi|1805796   -------
gi|329915|   --T----
gi|1066557   -------
gi|1066567   -------
gi|2388526   --T----
gi|1066617   --T----
gi|1930119   --T----
gi|1183032   -TT----
gi|1197096   --A---T
gi|2809127   -------
gi|1009258   --T----
gi|1009266   -------
gi|1066565   -------
gi|1066577   -------
gi|1805846   -------
gi|1805851   -------
gi|1805853   A-A---T
gi|2465799   -GT----
gi|2605601   -------
gi|2809126   -------
gi|2809128   -------
gi|296246|   --A---T
gi|3152988   -GT----
gi|3152993   --T----
gi|3152997   -------
gi|474334|   --T----
```

FIG. 3B

```
gi|1009264   GCTCAA TGC
gi|1160327   ------ ---
gi|1438429   ------ ---
gi|1181831   ------ ---
gi|1197128   A---T- ---
gi|1805843   ------ -A-
gi|1930117   ------ -A-
gi|1066559   A---T- ---
gi|1066561   ------ ---
gi|1805796   ------ ---
gi|329915|   ------ ---
gi|1066557   ------ ---
gi|1066567   ------ ---
gi|2388526   ------ ---
gi|1066617   ------ ---
gi|1930119   ------ ---
gi|1183032   ------ ---
gi|1197096   A---T- ---
gi|2809127   ------ ---
gi|1009258   -----G ---
gi|1009266   ------ ---
gi|1066565   ------ ---
gi|1066577   ------ ---
gi|1805846   A----- ---
gi|1805851   ----G- ---
gi|1805853   A---T- --T
gi|2465799   ------ -A-
gi|2605601   ------ ---
gi|2809126   ------ ---
gi|2809128   ------ ---
gi|296246|   A---TG ---
gi|3152988   ------ -A-
gi|3152993   ------ ---
gi|3152997   ------ ---
gi|474334|   ------ ---
```

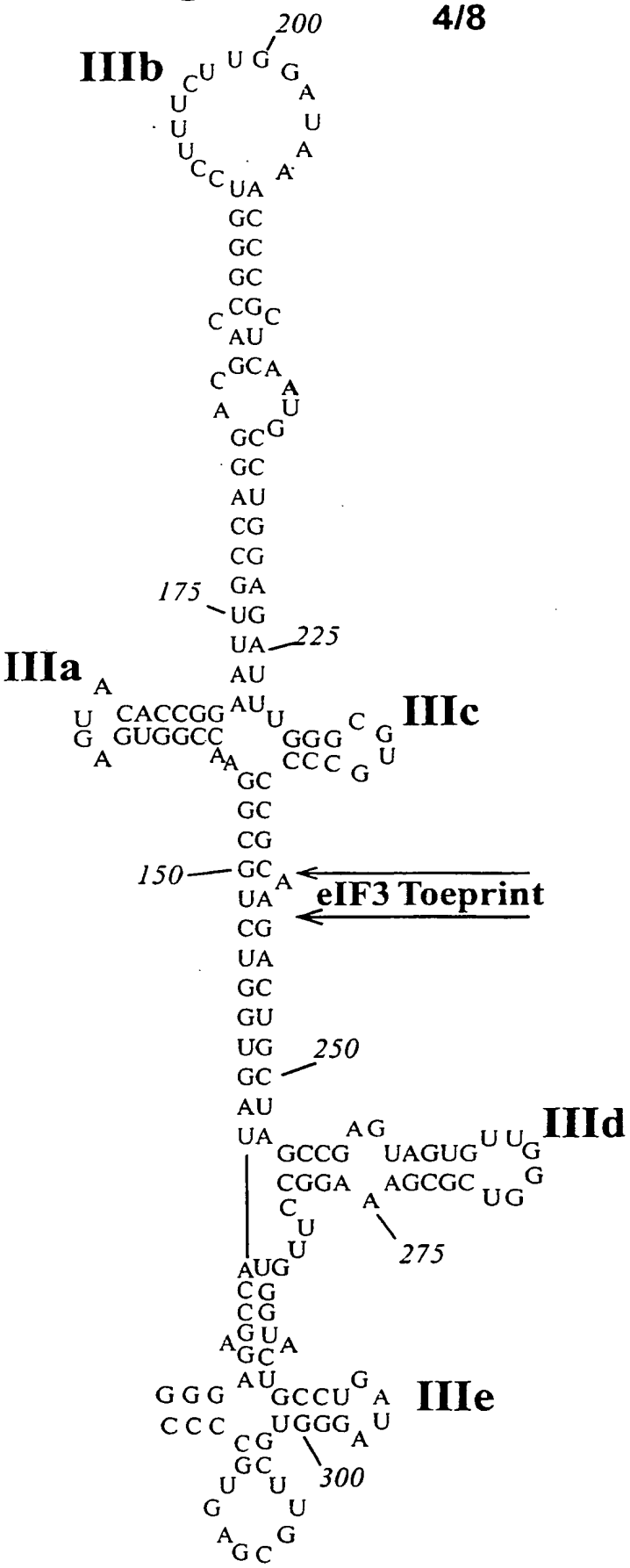


FIG. 5A

eIF3 (+++100%)
DIII (187nts)
(W/T + Mutants)

HCV 5 UTR Band shift probes

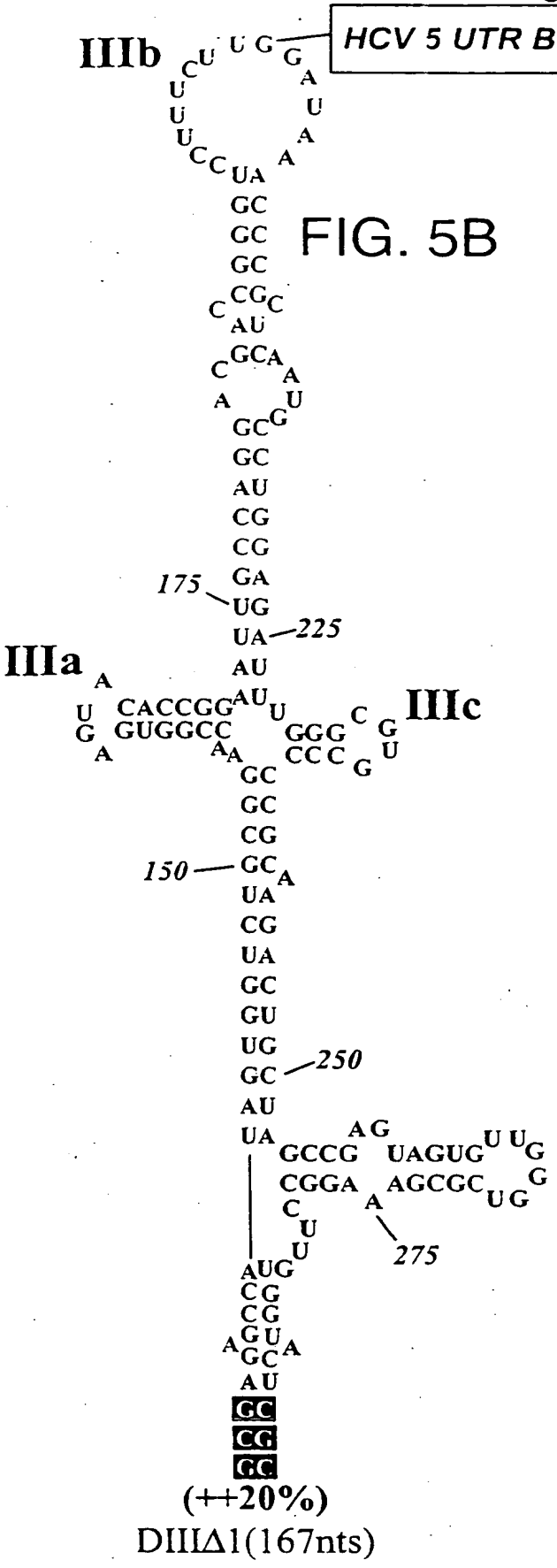


FIG. 5B

FIG. 5C

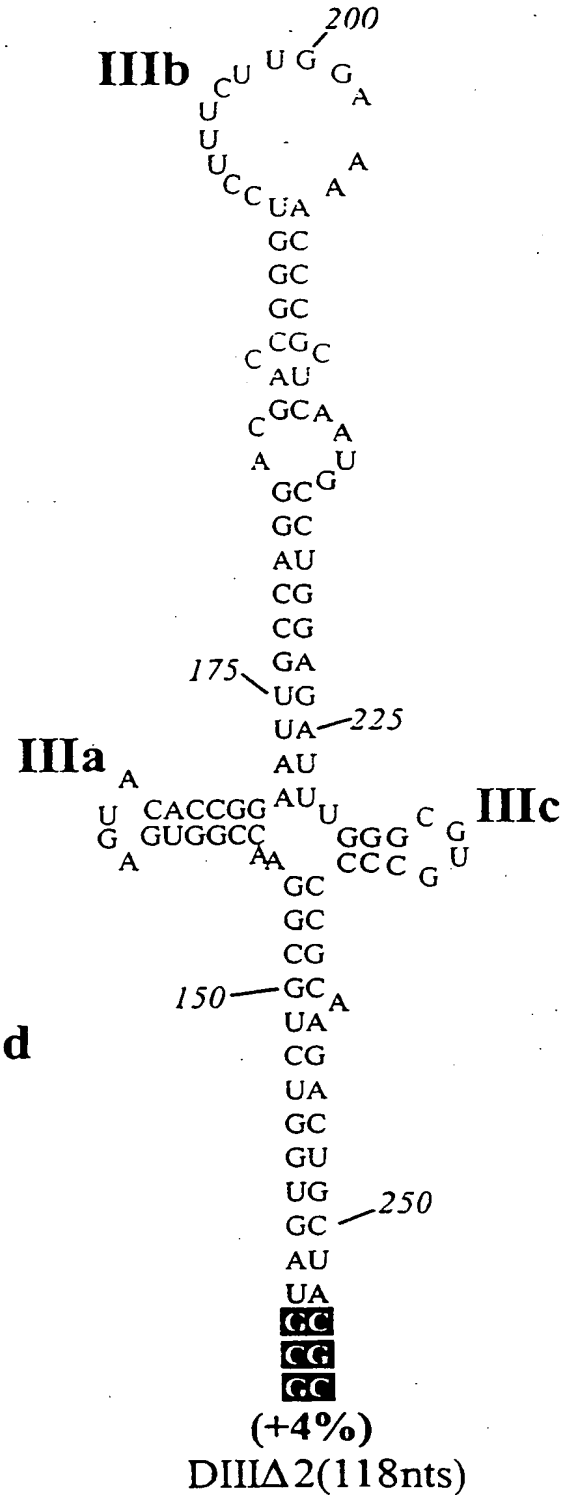
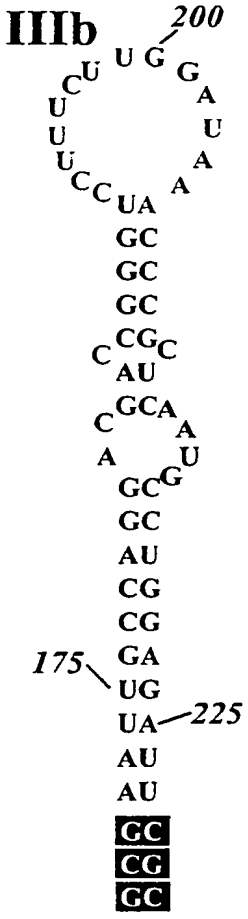
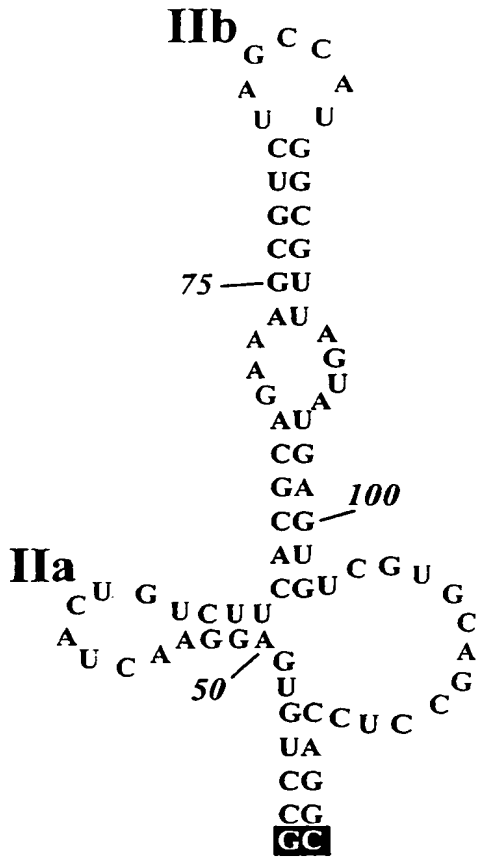


FIG. 5D



(-1%)
DIIIΔ3(62nts)

FIG. 5E



(-0.5%)
DII (78nts)

FIG. 7A

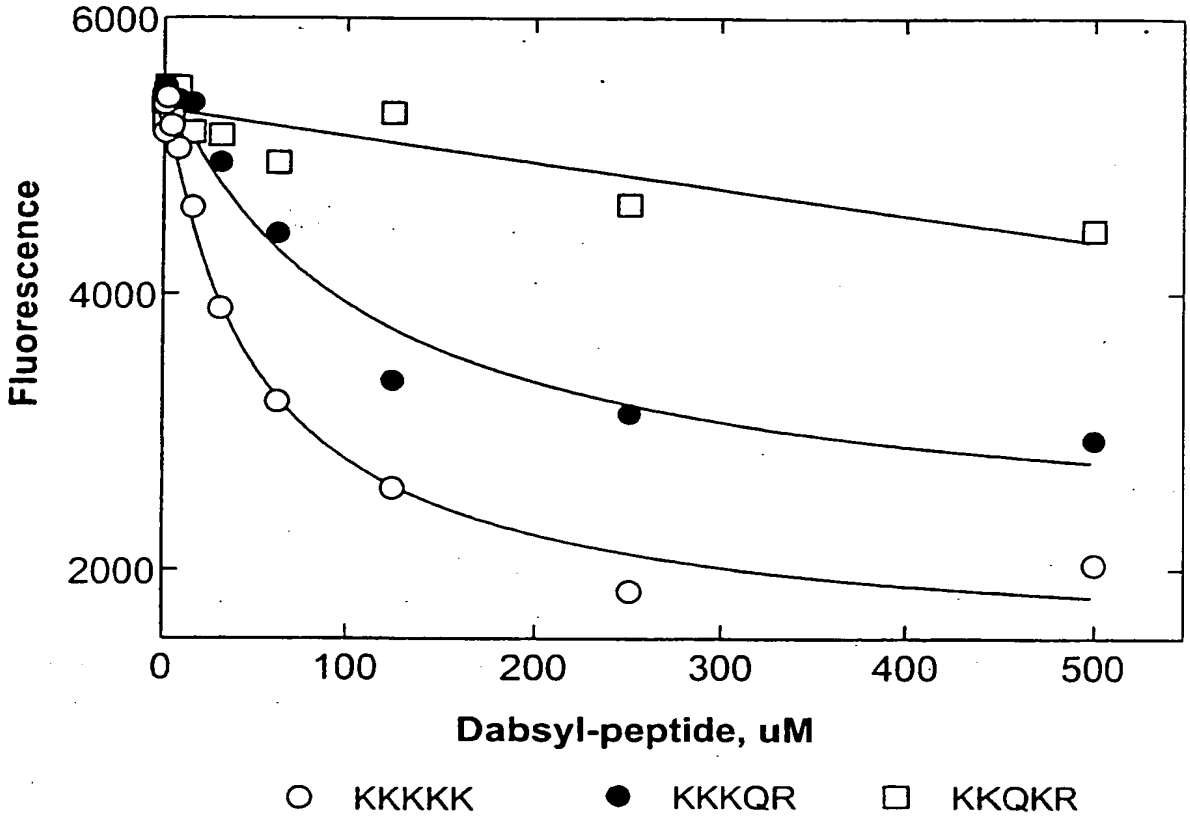
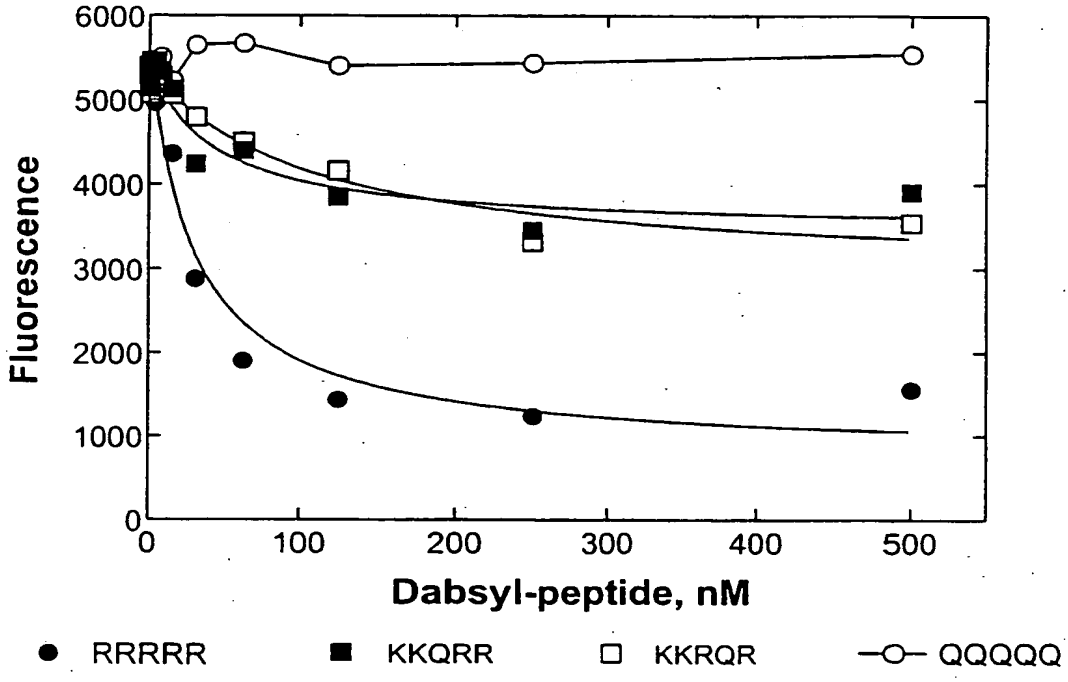
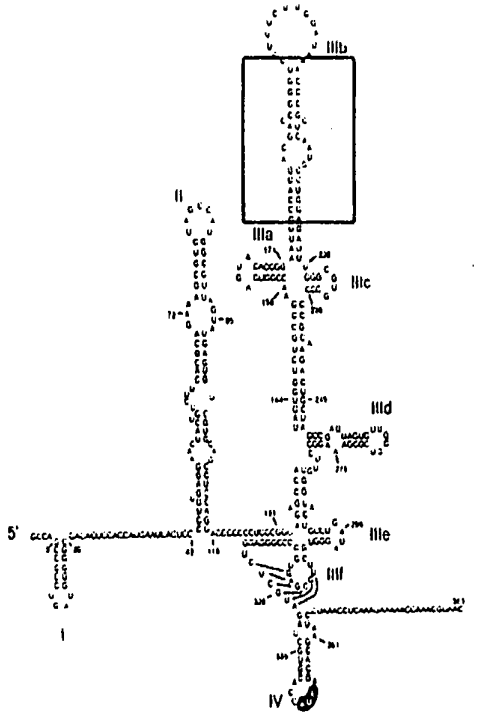
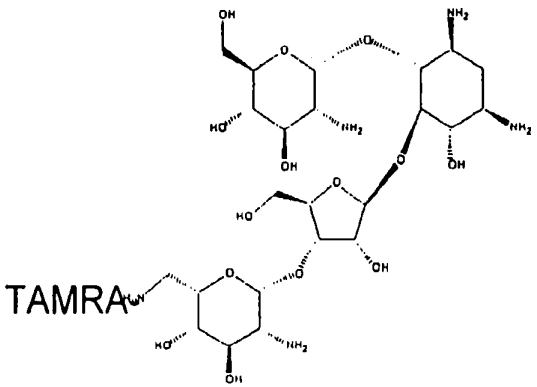

6000
4000
2000
Fluorescence
0
100
200
300
400
500
Dabsyl-peptide, uM
KKKKK
KKKQR
KKQKR

FIG. 7B


6000
5000
4000
3000
2000
1000
0
Fluorescence
0
100
200
300
400
500
Dabsyl-peptide, nM
RRRRR
KKQRR
KKRQR
QQQQQ





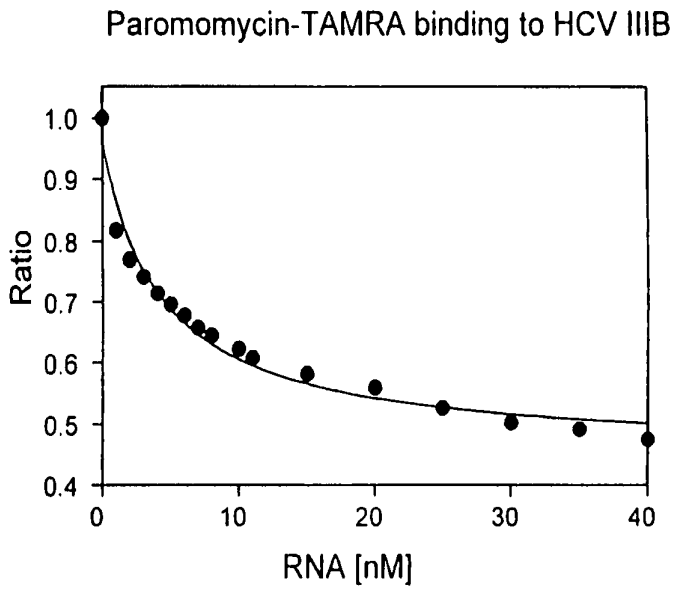


Fig 8A

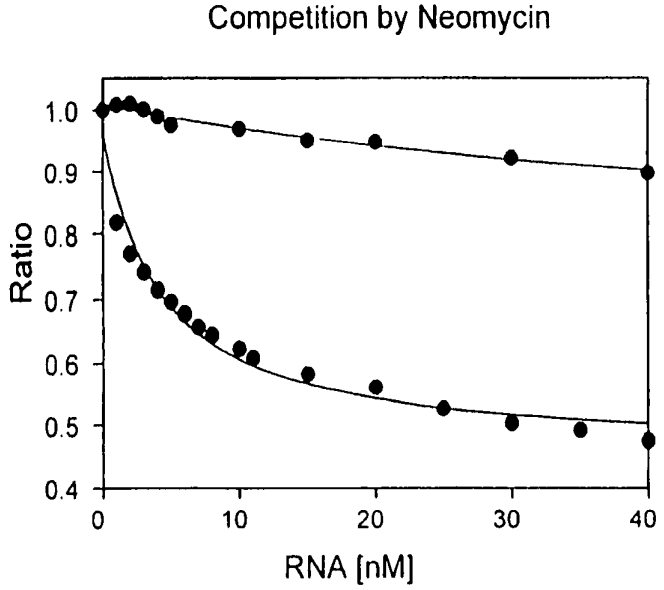


Fig 8B

Figure 8A and 8B

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(75) Inventors/Applicants *(for US only)*: **KARN, Jonathan** [GB/GB]; RiboTargets, Granta Park, Abington, Cambridge CB1 6GB (GB). **WALKER, Stephen** [GB/GB]; RiboTargets, Granta Park, Abington, Cambridge CB1 6GB (GB).

(74) Agents: **MASCHIO, Antonio** et al.; D Young & Co, 21 New Fetter Lane, London EC4A 1DA (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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WO 01/44266 A3

(54) Title: NUCLEIC ACID COMPOUNDS AND SCREENING ASSAYS USING THE SAME

(57) Abstract: The invention provides a compound comprising nucleotide sequences: -5'-P-N^1-N^2-G-N^3-C-I-3' and 3'-Q-N^4-N^5-N^6-N^7-N^8-N^9-Y-J-5' wherein P and Q are any two nucleotides that can form a Watson-Crick base pair, I and J are any two nucleotides that can form a base pair, N^1 and N^4 are not both C, when N^2 is A, N^7 is not G, N^9 can only be U if N^3 is A, when N^3 is G, N^9 is A, and wherein said sequences are capable of annealing, characterised in that said compound comprises 200 or fewer nucleotides.

INTERNATIONAL SEARCH REPORT

[redacted] International Application No
[redacted]/GB 00/04862

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07H21/00 C12Q1/68 C12Q1/70 G01N33/50 G01N33/58

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q G01N C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
MEDLINE, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SIZOVA ET AL.: "Specific interaction of eukaryotic translation initiation factor 3 with the 5' nontranslated regions of hepatitis c virus and classical swine fever virus RNAs" JOURNAL OF VIROLOGY, vol. 72, no. 6, June 1998 (1998-06), pages 4775-4782, XP000993423 page 4778 -page 4782; figure 4 ---	1-8,18, 19,22
X	DATABASE EMBL 'Online! EBI; ACC. NO.: AJ234462, 30 October 1998 (1998-10-30) MICHALEK ET AL.: "Hordeum vulgare genomic DNA fragment; clone MWG0065.rev" XP002178313 abstract --- -/--	1-8

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
25 September 2001	09/10/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	van Klompenburg, W

1

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International Application No
T/GB 00/04862

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WANG Y ET AL: "Specificity of aminoglycoside binding to RNA constructs derived from the 16S rRNA decoding region and the HIV-RRE activator region" BIOCHEMISTRY, AMERICAN CHEMICAL SOCIETY. EASTON, PA, US, vol. 36, 1997, pages 768-779, XP002120808 ISSN: 0006-2960 chart 1 figures 1-9 ---	18-22
A	WO 97 09342 A (SCRIPTGEN PHARM INC) 13 March 1997 (1997-03-13) page 23 page 34, line 3 - line 28 claims 1-9 ---	18-23
A	WALLIS ET AL.: "In vitro selection of a viomycin-binding RNA pseudoknot" CHEMISTRY AND BIOLOGY, vol. 4, no. 5, 1997, pages 357-366, XP001024489 abstract; figures 3,6 ---	1-23
A	US 5 922 857 A (SUH BYUNG S ET AL) 13 July 1999 (1999-07-13) claims 2-11; figures 2-4; examples 1-10 -----	1-23

INTERNATIONAL SEARCH REPORT

[redacted]ormation on patent family members

[redacted]ational Application No
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